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THE EFFECT OF SODIUM SULPHAQUINOXALINE AND SODIUM SULPHAMEZATHINE IN INTERRUPTED SCHEDULES OF TREATMENT ON THE DEVELOPMENT OF *EIMERIA TENELLA*

By

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INTRODUCTION

Following the original observation of Wehr and Farr (1947) that delayed bleeding might follow the use of Sulphamezathine for the control of caecal coccidiosis, Kendall and McCullough (1952) suggested that the drug caused temporary inhibition of the earlier stages of the parasite and that, unless this effect was reinforced by the developing immunity of the chick, withdrawal of the drug was likely to allow resumption of the life-cycle with possible sequelae of haemorrhage and death.

It appears that the very earliest stages of development of the parasite were affected only by very high drug concentrations (e.g. one per cent Sulphamezathine in the food). At lower concentrations, as will be shown later, development during the first 24 hours following infection is not significantly affected. On the other hand it appears that treatment covering the 24th to 48th hour following infection has an inhibitory effect which is indicated by a delay of up to 24 hours in the time at which mortality occurs as compared with the mortality with untreated birds. Similarly, treatment extending from the 24th to the 72nd hour following infection further inhibits development, mortality being delayed for as long as 48 hours.

At the 72nd hour of the normal life cycle the second generation schizonts are developing. Several authors (Horton-Smith and Taylor, 1945; Farr and Wehr, 1947; Cuckler and Ott, 1947) have shown that at this stage in its development the parasite is particularly susceptible to established treatment either with Sulphamezathine or with sulphaquinoxaline. In clinical practice, however, the results of treatment initiated at this stage are likely to be irregular, particularly in heavy infections. This is not only because the irregularity of the rate of development of individual parasites which is usually observed may lead to a significant number becoming mature before treatment is effective, but also because the partial inhibition of parasites may be insufficient to prevent fatal haemorrhage if their number is particularly large. To be fully effective therapy must precede the appearance of the developmental stage normally present at the 72nd hour of the uninhibited life cycle. During this developmental period even a slight alteration in the time at which treatment commences may induce a disproportionately large change in the mortality rate.

The effect of concentrations of sulphaquinoxaline and Sulphamezathine equivalent to those ordinarily used for the therapeutic control of the disease on different developmental stages of the parasite may be summarised as follows:

1. There is a negligible inhibitory effect on the first 24 hours development.

2. There is a considerable degree of inhibition between the 24th and 72nd hours.

3. The very marked effect on the 72nd and 96th hour stages of development will vary with the exact time at which treatment commences and with the weight of infection.

4. Treatment undertaken from the 48th to the 96th hour may be especially satisfactory since some degree of developing immunity will reinforce the inhibitory action of the drug.

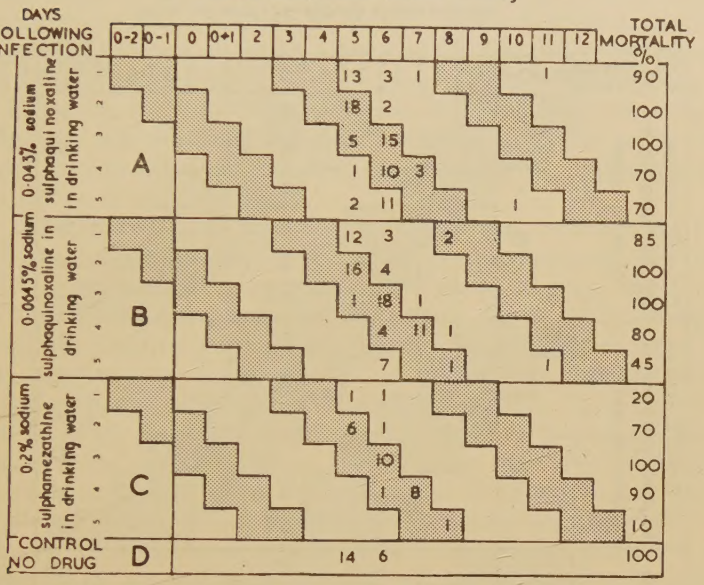
This paper records the results of treating chicks, experimentally infected with *Eimeria tenella,* with the sodium salts of Sulphamezathine and sulphaquinoxaline for intermittent periods. The observations are discussed in relation to the assumed effect of the drug on the various developmental stages of the parasite.

METHODS AND RESULTS

R.I.R. chicks were kept in isolation until required for experimental purposes at approximately one week old when they were moved to electrically heated, wire-floored hovers. Normally the chicks received a standard ration and tap water, but medicated water was substituted during periods of treatment. Observations were made on the sodium salt of sulphaquinoxaline at concentrations in the drinking water of 0.0645 per cent and 0.043 per cent and the sodium salt of Sulphamezathine (sulphadimethylpyrimidine) at a concentration of 0.2 per cent. Experimental infections were initiated by a single inoculation of approximately 100,000 sporulated oöcysts of *Eimeria tenella* directly into the crop, this number having previously been found to produce a consistently high mortality in the absence of treatment. In all observations the mortality rate was the sole criterion by which the success of therapy was judged. Deaths occurring in groups of 10 or of 20 chicks were recorded daily as shown in Tables 1 and 2. The final column in each Table indicates the total mortality as a percentage of the chicks in each group. Table 1 shows the effect of a 2-3-2-3-2 schedule of treatment i.e. three two day periods of treatment with intervals of three days without treatment, while Table 2 shows the results of a 3-2-3 schedule i.e. two three day periods of treatment with a two day interval without treatment. In both instances the mortality among chicks receiving sodium sulphaquinoxaline at concentrations of 0.0645 per cent and 0.043 per cent is compared with that among chicks receiving 0.2 per cent sodium sulphamezathine and with the mortality in a group of untreated birds.

TABLE I. SHOWING MORTALITY OCCURRING WITH 2-3-2-3-2 INTERRUPTED THERAPY

shaded areas denote periods on drug.



As indicated in the Tables all chicks received the experimental infection on the same day (Day O). Successive groups (numbered one to five or six in each of the Sections A, B, C, E, F, and G) were subjected to treatment on successive days. Thus in the first group of each section treatment commenced 48 hours before infection, while the second group received treatment 24 hours before infection and so on. Group 5 (Tables 1 and 2) received initial treatment 48 hours following infection and group 6 (Table 2) 72 hours following infection. The chicks in Sections D and H acted as untreated controls.

By this procedure it was possible to assess the effect of treatment which commenced at all stages in the development of the parasite.

INTERPRETATION OF DATA

Table 1 (2-3-2-3-2 schedule)

Development of the parasites in groups A_1, B_1 and C_1 of Table 1 cannot have been influenced by the initial period of medication which terminated on the morning of inoculation. The second period of dosing commenced, therefore, when the parasites had reached the 72nd hour of normal development. Under these circumstances there was little effect on the course of the disease

TABLE.2. SHOWING MORTALITY OCCURRING WITH 3-2-3 INTERRUPTED THERAPY.

shaded areas denote periods on drug

DAYS FOLLOWING INFECTION		0-2	0-1	0	1	2	3	4	5	6	7	8	9	TOTAL MORTALITY %
0·043% sodium sulphaquinoxaline in drinking water.	E	1						1	8					90
		2							6	3				90
		3							4	1	1	1		70
		4							2	3	3		1	90
		5							3	1				40
		6							7					70
0·0645% sodium sulphaquinoxaline in drinking water.	F	1							8	1				90
		2							4	5				90
		3								2	4			60
		4												0
		5							3					30
		6							9					90
0·2% sodium sulphamezathine in drinking water.	G	1							6	6				60
		2							1	7				40
		3									4	5		45
		4										1		5
		5												0
		6							4	1	1			30
CONTROL NO DRUG	H								10					100

following the use of sodium sulphaquinoxaline at either concentration but satisfactory results followed the use of sodium sulphamezathine. It may be inferred that under these circumstances Sulphamezathine reached an effective blood concentration more rapidly than did sulphaquinoxaline. In groups A_2, B_2 and C_2 there was similarly no effective inhibition of the parasite following the first period of treatment which covered only the first 24 hours development and during which, the parasite is resistant to the drug at these concentrations. Since the second period of treatment commenced when the parasites had developed beyond the 72nd hour stage, treatment was ineffective and mortality in all groups was high, deaths occurring on the same day as with the uncontrolled disease. In the third group of each section treatment included the period

of development from the 24th to 48th hour so that some inhibition of the parasite occurred. Seventy-two hours withdrawal of the drug was sufficient, however, to induce total mortality although the majority of the chicks died 24 hours later than those suffering from the uncontrolled infection.

Treatment covering the 24th to 72nd hour (the fourth group of each section) induced a delay in mortality of a further 24 hours i.e. most deaths occurred 48 hours after those in the untreated group D. At the lower concentration of sodium sulphaquinoxaline (0.043 per cent), however, the inhibition as the result of the first treatment was considerably less marked, one death occurring on the same day as with the uncontrolled disease and the majority of deaths occurring after a delay of only 24 hours.

In the groups A_5, B_5 and C_5 treatment was commenced at the 48th hour following infection. After termination of the initial period of treatment development of the parasites followed a course which varied with the particular drug used. In group A (0.043 per cent sodium sulphaquinoxaline) inhibition was incomplete, some deaths occurring on the same day as in the uncontrolled disease, while the majority of the birds died 24 hours later. At a concentration of 0.0645 per cent (group B_5) sodium sulphaquinoxaline exerted a more marked effect. There was a mortality, delayed by 24 hours as compared with the uncontrolled infection, but some of the birds survived. With sodium sulphamezathine (groups C_5) a high degree of survival was assured because the first period of drug administration was sufficient to control the infection until the resistance of the chick had developed.

Table 2 (3-2-3 schedule)

Consideration of this Table (using the same criteria for assessing the results as in Table 1) shows that in most instances the effect of the 3-2-3 type of interrupted treatment was to allow development of the parasites to proceed to about the 72nd hour of development by which time the second period of treatment became operative.

The final result will depend upon the exact stage to which the second generation schizonts have developed by the time the second period of drug administration commences and partly on the speed with which an effective drug concentration is attained.

In the first group of each section E, F and G, the initial period of treatment terminated 24 hours after infection so that no inhibition of development could be expected. The second period of treatment became effective therefore, after the 72nd hour of development had been reached; there was a moderate therapeutic effect with sodium sulphamezathine but a negligible effect with sodium sulphaquinoxaline.

Treatment covering the first 48 hours following infection (the second group of each section) caused inhibition sufficient to delay mortality by 24 hours with sodium sulphaquinoxaline and with

sodium sulphamezathine there was a reduction in mortality to 40 per cent. Treatment starting at the time of infection caused inhibition of development, shown most characteristically in the third group of section G 24 to 48 hours later. Such delay allowed the second period of treatment to control mortality to 70 per cent with 0.043 per cent sodium sulphaquinoxaline, 60 per cent with 0.0645 per cent sodium sulphaquinoxaline and to 45 per cent with sodium sulphamezathine. When the initial period of treatment covered the 24th to 96th hour following infection, the parasites had not developed as far as the normal 72nd hour stage. Control was excellent with sodium sulphamezathine and at the higher concentration of sodium sulphaquinoxaline but unsatisfactory with the lower concentration of the latter. When the first period of treatment covered the 48th hour to the 120th hour following infection (the fifth group of each section) the best therapeutic effect was obtained as the drug had an effective action for the entire initial period of treatment. Finally, in the sixth group of each section (E, F and G), the initial period of therapy commenced at the termination of 72 hours uninhibited development. Deaths occurred in each group on the same day as with the uncontrolled disease but with sodium sulphamezathine considerable therapeutic effect was apparent.

DISCUSSION

It appears that any form of interrupted therapy which includes withdrawal of the drug for a period exceeding two days is unlikely successfully to control mortality. No evidence of any inhibitory effect on the first 24 hours development of the parasite could be found. The summation of this period with any period without drug which exceeds 48 hours will allow development to advance beyond the point corresponding to the normal 72nd hour stage; the development of the second generation schizonts is already well established and a further period of treatment is likely to be ineffective. Interrupted therapy using the 2-3-2-3-2 schedule can be effective only when the period of drug administration happens to control the early second generation schizont. The degree of control will be related to the speed with which the drug attains an effective blood level.

The 3-2-3 schedule of interrupted therapy, on the other hand, offers considerable advantages in that the selective effect of the drug ensures that the majority of the parasites are in the earliest phase of the second generation schizont at the time a period of treatment becomes effective. Under such circumstances control of the disease is assured in a high proportion of chicks.

CONCLUSIONS

In experimental coccidiosis in chickens interrupted schedules of treatment were compared using sodium sulphaquinoxaline at concentrations of 0.043 per cent and 0.0645 per cent, and sodium

sulphamezathine at a concentration of 0.2 per cent of the drinking water.

The type of interrupted treatment in which the drug was given on two successive periods of three days with a two day period without treatment proved more effective in limiting development of the second generation schizont than two day periods of treatment with intervals of three days.

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STREPTOCOCCAL METRITIS IN THE MINK

By

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INTRODUCTION

A mink breeder reported that twenty pregnant females had died at the end of gestation and many others were believed to have aborted although no aborted foetuses had been found. In May, 1953 four carcases were received from him. No clinical history was available as it was thought that the animals had retired to their dens for parturition and it was the breeder's practice not to interfere with them under these circumstances. Thus it was only after some days that the dead animals were found. It was not known whether the animals had given birth to dead litters and eaten the foetuses and the membranes or whether these were absorbed in an earlier stage. On post-mortem examination purulent metritis was found in three cases. In one a macerated foetus was present and in another the metritis was accompanied by a purulent peritonitis. In all three the spleen was enlarged and swollen. Smears of the uterine contents and from other organs contained Gram-positive cocci. Pure cultures of an α streptococcus were isolated from heart, liver, spleen, kidney, uterus, peritoneal cavity and brain. The fourth specimen did not relate to the outbreak. It was a non-pregnant female without lesions of the uterus that had died from pneumonia and from it no micro-organisms were isolated.

BACTERIOLOGICAL FINDINGS

The streptococci isolated were of ovoid form and occurred in pairs and short chains of 4 to 6 organisms. They were non-motile and Gram-positive. There was no liquefaction of gelatin. On serum-agar growth occurred as small transparent colonies while growth in serum-broth was slightly turbid without sediment. Colonies of horse-blood-agar were surrounded by narrow greening zones (α haemolysis). In litmus-milk acid was formed followed by curdling after eight days. In Warren and Crowes medium acid was formed from glucose, mannose, fructose, lactose, maltose, saccharose, raffinose and trehalose, whereas salicin, arabinose, xylose, rhamnose, inulin, dextrin, glycerol, mannitol, sorbitol, dulcitol and inositol were not fermented. Indol was not formed and nitrite was not produced from nitrate. After some generations on artificial media (serum-agar, and serum-broth) all strains died before there had been an opportunity for serological typing.

EXPERIMENTAL

For use in experimental animals a primary culture from the spleen of a natural case was used. This culture was grown in serum-broth for 24 hours at 37°C. In all cases infection was by subcutaneous injection.

(a) *2 mice, dose 0.1 ml.* One died after two days and α streptococci were isolated from heart, liver, spleen and kidney. The other mouse survived.

(b) *2 pregnant guineapigs, dose 0.1 ml.* Both animals died after seven days. One had aborted on the day prior to death and the other died during abortion; α streptococci were isolated from heart, liver, spleen, kidney, uterus and brain of both animals and from the foetuses.

(c) *A pregnant ferret, dose 1 ml.* The animal died two days after inoculation. At post-mortem the spleen was found to be very enlarged and swollen and α streptococci were isolated from heart, liver, spleen, kidney, foetuses and placentae.

(d) *A non-pregnant ferret, dose 1 ml.* This animal survived without signs of illness.

The strains isolated from these animals fully resembled the description given above.

DISCUSSION

Nothing was found in the literature available relating to the aetiology of metritis and abortion in the mink. Kennedy (1949) reported that streptococcal infection may occur in these animals but no details were given. The streptococcal infection described by Hartsough (1951) was a disease of young animals caused by β hemolytic streptococci. Thus the strains of α streptococci obtained from this outbreak could not be compared with descriptions in the literature and their cultural characteristics differed from those of the species described by Bergey (1948). Although no minks were used in the experimental infections it can be concluded from the results obtained that Koch's postulates were fulfilled and that the α streptococci which were isolated are a cause of metritis and abortion in the mink.

CONCLUSIONS

Pure cultures of an α haemolytic streptococcus were isolated from three cases of spontaneous metritis in mink. The organism was not isolated from another mink from the same farm which died from another cause.

In pregnant guinea-pigs injection of the organism produced abortion.

In the ferret the organism was only pathogenic for the pregnant animal and although this animal did not abort the organism was isolated from the foetuses and placentae.

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THE GOBLET CELLS OF THE LARGE COLON OF THE HORSE

By

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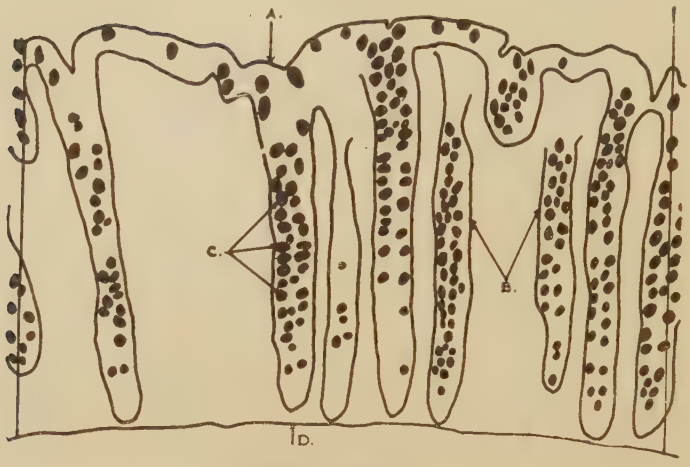
INTRODUCTION

It has been shown by Alexander, Macpherson and Oxford (1952) that the contents of the right dorsal colon of the horse were rich in a mucoprotein-like substance. Since this substance must have been added to the colon contents after their passage through the ventral colon it seemed reasonable to suppose that it had been secreted by the mucous membrane of the dorsal colon. A histological investigation was therefore undertaken to determine whether any difference existed between the mucous membrane of the ventral and dorsal colons.

MATERIALS AND METHODS

Pieces of intestinal wall about 1½ inches square were removed from the right dorsal (first part of large colon) and right ventral colons (last part of large colon) of six freshly killed horses. Those from the right ventral colon were taken from an area approximately 1½ feet from the base of the caecum and those from the right dorsal colon from the region about 1½ feet from its junction with the small colon. In some cases, pieces from the walls of the caecum and small colon were taken for comparison. The tissues were then fixed in Heidenhain's Susa and paraffin blocks prepared by the usual method. The sections cut from the blocks were of uniform thickness (6 microns), and were stained by (1) haematoxylin and eosin and (2) Masson's trichrome. The stained sections were used to count the number of goblet cells present in approximately equal lengths of mucous membrane and for measurements of the average thickness of the mucous membrane. A portion of each section was projected on to a sheet of graph paper, the image being a magnification of 300 (see Figs. I & II). As far as possible, areas of mucous membrane in which the muscularis mucosae did not show folding, and in which the intestinal glands were cut longitudinally, were chosen (Figs. III & IV). The outline of the projected image showing the intestinal glands was traced on to graph paper and the position of each goblet cell indicated (Figs. I & II). The number of goblet cells visible in a piece of mucous membrane lying within the length (25cm) covered by ten large squares on the graph paper were counted. The photographs (Figs. III & IV) are included to show the appearance of actual histological sections of the right dorsal and right ventral colons. The thickness of the mucous membrane was

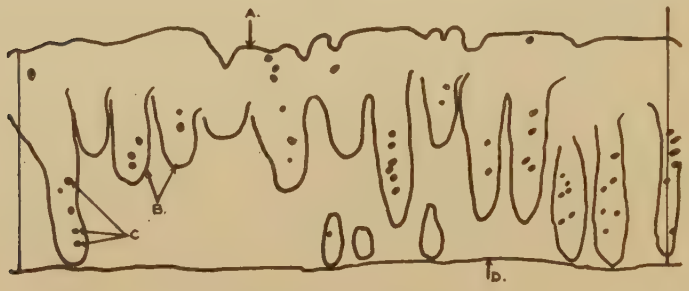
Fig. I



A. SURFACE OF EPITHELIAL LINING.
B. INTESTINAL GLANDS.
C. GOBLET CELLS
D. MUSCULARIS MUCOSAE

Horse V
Right Dorsal Colon
Number of Goblet Cells=250. Length of Muscularis Mucosae 0.087cm. Average Thickness of Mucosa 0.052cm. X 300.

Fig. II



A. SURFACE OF EPITHELIAL LINING.
B. INTESTINAL GLANDS.
C. GOBLET CELLS.
D. MUSCULARIS MUCOSAE

Horse V
Right Ventral Colon
Number of Goblet Cells=47. Length of Muscularis Mucosae 0.084cm. Average Thickness of Mucosa 0.029cm. X 300.

measured at intervals of 2.5cm on the projected image, the measurements being made from the inner surface of the muscularis mucosae to the free border of the epithelial cells. The lengths of the pieces of mucous membrane examined were measured by following the

course of the muscularis mucosae projected on the graph paper with a cartometer. Four areas of mucous membrane were examined from the right dorsal and right ventral colons of each of the six horses.

RESULTS

The average length of the actual projection of the mucous membrane on the graph paper at a magnification of 300 was 25.74cm and within a range of 25.0 to 27.0cm. Correction of the number of cells for this slight variation in apparent length of the section counted was on the basis of simple proportion. The averages of the counts of goblet cells in this 25.74cm apparent length (0.083cm true length) are shown in Table I. Analysis of variance indicated that the difference in the average count of goblet cells in the right dorsal colon as compared with the right ventral colon is significant at the

TABLE I

AVERAGE NUMBER OF GOBLET CELLS

In 0.083 cms length of mucous membrane from right dorsal and right ventral colons

Area	*I*	*II*	*III*	*IV*	*V*	*VI*	*Mean*
Right dorsal colon	162	81	132	78	207	121	130
Right ventral colon	42	77	40	41	51	97	58

TABLE 2

THE THICKNESS IN CENTIMETRES (X300) OF MUCOSA

At site of count of goblet cells

Area	*I*	*II*	*III*	*IV*	*V*	*VI*	*Mean*
Right dorsal colon	12.78	9.13	11.64	12.17	14.45	13.03	12.20
Right ventral colon	6.70	12.06	6.84	9.49	9.61	9.92	9.10

5 per cent level. In order to determine whether this difference was due to the difference in thickness of mucous membrane a comparison of mucosal thickness was carried out (Table 2). Analysis of variance shows that the difference in average thickness (12.20cm compared with 9.10cm) fails to reach the 5 per cent level of significance. The numbers of goblet cells were corrected for variation in thickness of the mucosa by analysis of co-variance, and the corrected means unaffected by variation in thickness are as shown in Table 3. Analysis of co-variance showed that after adjustment for variation

in thickness, the average number of goblet cells of the right dorsal colon is significantly greater than the average number in the right ventral colon.

TABLE 3

NUMBER OF GOBLET CELLS

After adjustment for difference in thickness of mucosa

Area	*I*	*II*	*III*	*IV*	*V*	*VI*	*Mean*
Right dorsal colon	131.1	103.0	117.6	63.6	152.8	86.5	109.1
Right ventral colon	99.3	56.5	95.3	57.8	66.1	107.6	80.4

DISCUSSION

Animals capable of digesting cellulose provide a fermentation chamber in some part of their digestive tract. In the horse the large intestine is greatly developed for this purpose, and is divided into four parts, the caecum, large colon, small colon and rectum.

From recent work it appears that the large colon can be subdivided functionally into at least two parts. It had been demonstrated by Alexander (1952) that "The separation of the fatty acids showed that more propionic acid was produced in the ventral than in the dorsal colon, and more butyric and higher acids in the dorsal than in the ventral colon", and also that the rate of cellulose digestion is more rapid in the ventral than in the dorsal. Alexander *et al* (1952) found that liquor from the right ventral and right dorsal parts of the colon differed, the liquor from the right dorsal colon being thicker and darker in colour and containing more of a muco-protein-like material. Adam (1951) showed that the concentration of ciliate protozoa varied in the different regions of the large intestine and that two distinct populations of ciliates occurred in the large intestine of the horse, one from the caecum to the pelvic flexure and the other from the pelvic flexure to the rectum.

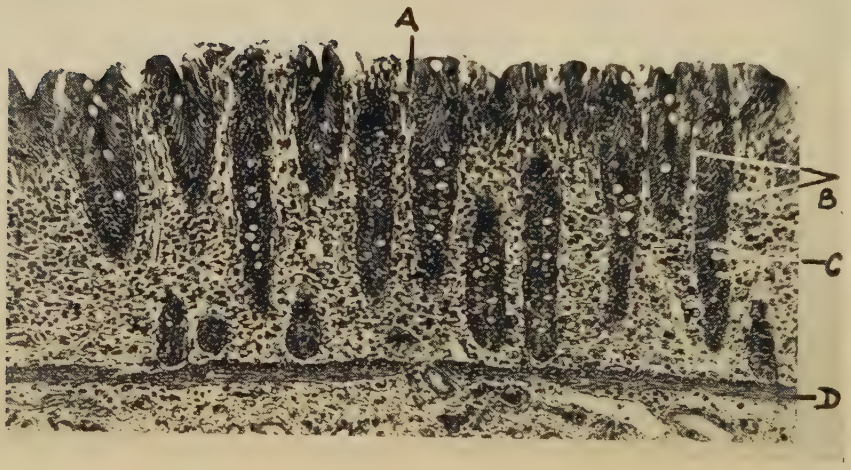
The histological examination of the mucous membranes of the right dorsal and ventral colons revealed a considerable difference in structure between them giving anatomical support to the other differences. The large number of goblet cells present in the right dorsal colon probably explains the greater quantity of muco-protein material present in that part of the colon; moreover not only are the goblet cells more numerous in the right dorsal colon but they appear larger and more active than those of the right ventral colon (Figs. III & IV). It is possible also that they produce different types of secretion. It would be of interest to study the mucous membranes of the left ventral and left dorsal colons to determine if there is a similar difference in these two parts.

Fig. III

Right Dorsal Colon (Horse V) x 250.

A. Surface of Epithelial lining.
B. Intestinal glands.
C. Goblet cells.
D. Muscularis mucosae.

Fig. IV



Right Ventral Colon (Horse V) x 250.

A. Surface of Epithelial lining.
B. Intestinal glands.
C. Goblet cells.
D. Muscularis mucosae.

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CONCLUSIONS

There are significantly more goblet cells present in the right dorsal than in the right ventral colon of the horse.

ACKNOWLEDGMENT

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EXPERIMENTAL INFECTION WITH *MYCOBACTERIUM JOHNEI*

I. The Effect of a Low Calcium Diet on the Disease in Goats

By

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INTRODUCTION

Marek and Wellmann (1931) observed that goats were highly susceptible to calcium deficiency diseases. Glock, Murray and Glover (1939) showed that a calcium deficiency can be induced in kids by feeding diets low in calcium and vitamin D and that an acute osteo-dystrophy is likely to occur. Kids are also suitable animals for experimental investigations on Johne's disease and can be infected by the mouth or by intravenous injection (M'Fadyean and Sheather, 1916; Levi, 1948). The experiments described in this paper were undertaken to determine whether the course of the disease and the extent of the lesions in experimental infection in kids could be modified by a diet in which there was a low level of calcium and the Ca/P ration was significantly unbalanced.

METHODS

Animals. The kids used in these experiments were obtained from the Veterinary Laboratory, Ministry of Agriculture, Weybridge, and were about two months old when the experiments commenced. All kids were negative to the single intradermal johnin test at the beginning of the experiments. A total of 26 kids were used for the trials in three separate experiments. The first of these, involving ten kids, was started in November 1950, eight more animals were infected in June 1951, and the last group of eight in May 1952.

Accommodation. A large animal house containing a single row of pens was used to accommodate the kids, a pair of kids occupying each pen. The pen dimensions were 5 ft. x 3 ft. 9 ins., with high separating walls of galvanised iron sheeting carried down to the concrete floor. Wood shavings provided the bedding and helped to seal the dividing walls thus preventing faeces and urine from gaining access to adjoining pens. Separate feed troughs, watering buckets and hay racks were used in each pen. The skylight and some of the windows were blacked out in order to prevent direct sunlight from reaching the goats.

Diets used. In each experiment the kids were divided into two groups. All the animals received the same basal diet, which was low in calcium, but half the animals, forming the control group, were given a calcium supplement and cod liver oil. In the third experiment the kids on the

calcium deficient diet had a special low calcium hay, while the controls were given the same quantity of normal hay. The three basal diets, which follow fairly closely those used by Glock, *et al,* are given in Table 1.

TABLE 1

BASAL DIET PER PAIR OF GOATS PER DAY

	Experiment 1	*Experiment* 2	*Experiment* 3
Hay	1 lb.	1 lb.	2 lb.
Flaked maize	1½ lb.	1 lb.	1 lb.
Ground or crushed oats	—	1 lb.	1 lb.
Bran	1½ lb.	1 lb.	—
Sodium chloride ..	—	*¾ oz.	½ oz.

*Not given for the first two months.

In the first two experiments the additional calcium was given in the form of calcium carbonate mixed with the food, each pair of kids received 16 g. daily. They also had approximately ½ oz. of cod liver oil three times a week. The calcium supplement for the third experiment was bone meal, 2 oz. being allowed for each pair of kids; ½ oz. of cod liver oil was mixed with the dry food on alternate days.

In an attempt to prevent the absorption of calcium, the kids on the calcium deficient diet in the second experiment, were given potassium dihydrogen phosphate. At first it was added to the dry feed, 12 g. being given to each pair of kids per day: the dose was gradually increased to 22 g. After two months the KH_2PO_4 was added to the drinking water, each pair of kids receiving 32 g. daily. No KH_2PO_4 was given to the kids used in the other experiments.

Calcium estimation. Blood samples were taken from the kids for calcium estimation every two to four weeks. The serum calcium was estimated by the method of Clarke and Collip (1925). Estimations were also made of the serum inorganic phosphorus levels of some of the kids, using the micro-method of King and Allott (1947).

Smear and culture examination. At the post-mortem examination the whole of the small intestine, caecum, and first part of the large intestine was opened and examined. Smears were made from the surface mucosa at intervals of about three feet, and examined for typical clumps of *Mycobact. johnei.* The number of clumps in 50 microscope fields was then counted. These counts were of necessity something of an approximation, as the number of organisms in the clumps varied from a pair of rods to a rather diffuse mass: in practice the clumps were counted regardless of their size. Where there were less than 50 clumps per field they were counted as

accurately as possible, but above that the numbers were estimated as between 50 and 100, or more than 100 clumps. The mean number of clumps was worked out for the 50 fields and plotted against their relative position in the intestine.

Portions of the liver, lung, spleen and kidney were taken from the carcases of most of the animals and cultured. Cultures were also made from the mesenteric, retropharyngeal and bronchial lymph nodes of all the kids, and in some cases from the submaxillary, hepatic and superior cervical lymph nodes, and from the tonsils. Pieces of tissues were also fixed in 10 per cent formol saline for section.

The material, after grinding with sand in a mortar, was filtered through four layers of butter muslin and treated with 5 per cent oxalic acid for 45 mins. at 37°C. It was then spun down and the deposit sown on to three slopes of egg yolk-phlei medium (Taylor, 1950). The slopes were examined for growth after six weeks and finally discarded after four months incubation.

RESULTS

Kids Infected by the Oral Administration of Natural Material

In all, 18 kids—in two separate experiments—were infected by repeated drenching with scrapings of gut mucosa from bovine cases of Johne's disease. The 10 kids, used for the first of these tests, were fed a diet consisting of flaked maize, bran and hay (Table I). After six months there was no significant drop in the serum calcium levels of the kids on the deficient diet and the experiment was terminated. The post-mortem findings on these animals are not considered in this paper.

The eight kids used for the second experiment were infected by drenching in the same way. The basal diet of these animals consisted of equal parts of flaked maize, bran, ground oats and hay (Table I). The deficient group was also given KH_2PO_4.

Of the four kids on the low calcium diet in this test three died within five months of the start of the experiment. One died after two months, as the result of an accident, and two between the fourth and fifth month. The death of one was possibly the result of hypocalcaemia, as its serum calcium level was 5·4 mg. four days before death; calcium borogluconate was given subcutaneously but the animal did not respond. The surviving kid (D.1940) was killed 41 weeks after its first infecting dose. Three of the control kids also died within five months; the cause of death of two was uncertain, but the other revealed a Salmonella infection. The survivor, (D. 1896) was killed after 39 weeks. For several weeks before its death this animal had been falling off in condition and had lost weight.

The animals taking part in these trials were weighed at fairly regular intervals, but there is little information to be obtained from

the weight records of the kids in this test owing to their early death. However, the one control kid (D.1896), in which clinical symptoms developed, did show a definite loss of weight.

The serum calcium levels of the two surviving kids were only determined for the first six months of the experiment. The value for D. 1940, on the low calcium diet, dropped to 7·0 mg. after 16½ weeks; at 20 weeks it was given a calcium supplement and at 24 weeks—when the serum was last tested—it had risen to 9·2 mg. Although maintained on the deficient diet for a further three months, this animal did not show any signs of calcium deficiency. After 24 weeks, the control kid, (D. 1896) had a serum calcium level of 11·3 mg.

Kids Infected by a Combination of the Oral and Intravenous Routes

In the experiment reported in this section, eight kids were infected by a combination of the oral and intravenous routes. Each animal received a drench of gut scrapings, and an intravenous injection of 10 ml. of a fluid culture of a strain of *Mycobact. johnei* recently isolated from an experimentally infected goat. The culture was grown in a modified Dubos medium (Glover, 1952). Both the drenching and intravenous injection were repeated after ten days.

The diet in this experiment was the same as for the second experiment (Table I) except that the bran was omitted and replaced by more hay, which was low in calcium for the deficient group. The kids on the low calcium hay were not given any KH_2PO_4.

Two of these eight kids died of coccidiosis six and seven weeks after their last infecting dose. As both these animals were in the low calcium group one of the controls (D. 1644) was transferred to the deficient diet to balance the numbers. Six kids remained and were killed in pairs of one deficient and one control animal. One pair, (D. 1665 and D. 1667) was killed 28 and 29 weeks after the start of the experiment, the second pair, (D. 1648 and D. 1650) after 36½ and 38 weeks, and the last two, (D. 1644 and D. 1669) after 39 and 40 weeks. Only one kid, (D. 1648) became a definite clinical case; it scoured intermittently for about a month before it was killed.

Weight

The weight records of the six animals are shown in Figs. 1 and 2. The loss of weight of D. 1667 and D. 1669, between the fourth and eighth week after infection, coincided with a period of scouring, probably due to coccidiosis. With the exception of D. 1669, all the kids reached their maximum weight by the 21st week. The subsequent loss of weight started rather earlier, and was most marked, in the clinical case D. 1648.

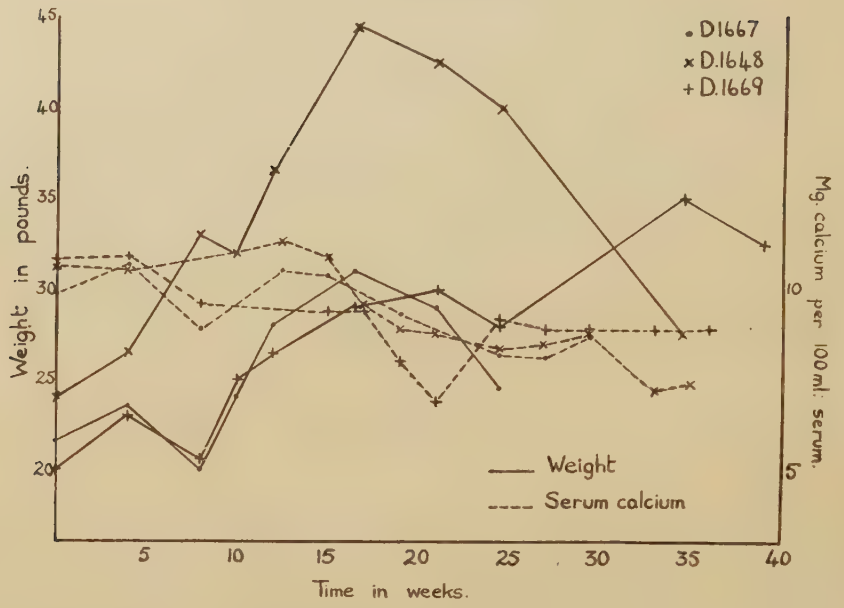

FIG. 1. KIDS ON THE CONTROL DIET.

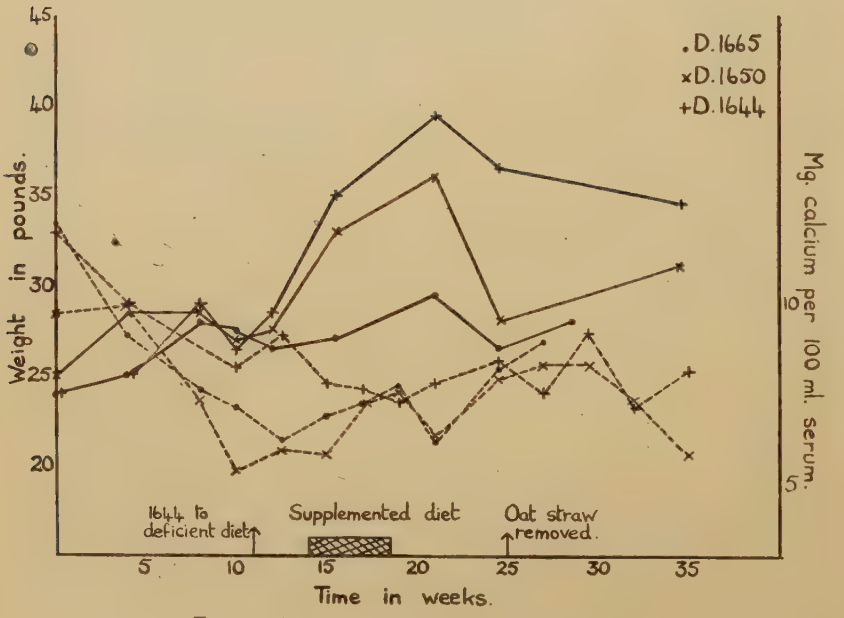

FIG. 2. KIDS ON THE LOW CALCIUM DIET

Serum Calcium Levels

For the first 15 weeks after infection the three animals on the control diet maintained a serum calcium level of between 9 to 12 mg./100 ml. which is within the normal range. (Coffin, 1945). They then began to show a small but definite drop. (Fig. 1). With one exception, all the animals had serum calcium values below 9·0 mg. from the 19th week until their death.

The two kids (D. 1650 and D. 1665) which received the low calcium diet from the start of the experiment, showed a fairly rapid fall in their serum calcium levels : by the 12th week they had dropped to 5·9 and 6·2 mg. (Fig. 2). At this stage there was a danger that the animals might succumb from low calcium tetany, so they were given the supplemented diet for about five weeks. This resulted in a rise in their serum calcium levels to 7·6 and 7·7 mg. When they were returned to the deficient diet there was an initial drop followed by a gradual rise which reached 8·3 and 9·0 mg. by the 27th week. This rise was later thought to be due to the consumption of oat straw, which had been provided as bedding at the start of the cold weather, since, when this was removed, the serum calcium values began to drop again. The third kid in this group, (D.1644) was transferred to the deficient diet about eleven weeks after the start of the experiment : by the 19th week its serum calcium had dropped to 7·3 mg. but it then rose to 9·2 mg. at the 30th week and was 8·2 mg. when last examined after 35 weeks.

In both groups of kids the inorganic phosphorus values showed considerable fluctuation. The values for normal goats vary, however, from 3·0 to 11·0 mg. per 100 ml. serum (Coffin), and all the values obtained with our control kids fell within this range. The clinical case, (D. 1648) had a consistently higher inorganic phosphorus level than the other two kids on the supplemented diet. From the 15th week—when the first of these determinations was made—until death at the 39th week, the mean value was 9·8 mg., while the means of the other two control kids were 5·3 and 7·5 mg. respectively. The Ca/P ratio for D. 1648 was abnormally low, generally being less than 1 : 1 instead of the normal 1 : 2. On the whole the animals on the deficient diet had higher levels of inorganic phosphorus than the controls, the means for the three kids being 9·0, 9·7, and 12·3 mg. One animal, (D. 1644) gave values of over 15 mg. on several occasions. The Ca/P ratios for the serum of the animals on the calcium deficient diet were low, generally less than 1 : 1.

Clinical Symptoms

One clinical case occurred in the control group in each of the second and third experiments (D. 1896 and D. 1648). Both kids began to fall off in condition eight months after the beginning of the experiments, but the deterioration was a slow process and there were no signs of actual physical weakness or incoordination when the

kids were killed, in each instance several weeks after the onset of symptoms. Diarrhoea was intermittent and at no time approached the profuse watery scour so often seen in clinical bovine cases of Johne's disease. One animal (D. 1896) developed a temperature of 104°F. soon after clinical symptoms appeared but this returned to normal after oral dosing with sulphamezathine.

Two of the kids (D. 1665 and D. 1650), in the calcium deficient group of the third experiment, began to show symptoms of osteodystrophy and general bone weakness about the time when their serum calcium levels showed marked falls below normal levels. This was evidenced in D. 1665 by a bowing of the hind legs, and a disinclination to stand. Kid D. 1650, presented similar signs and symptoms of bone weakness and dystrophy but to less marked degree.

Other animals in the experiments, at various times, had loose faeces and in some cases diarrhoea, but this was a rare occurrence and as a Salmonella organism was cultured from the intestines of one kid which died in the second experiment, and two kids died with acute coccidiosis in the third, the significance of this intermittent diarrhoea in relation to the Johne's infection is obscure.

Post-mortem Findings

Second Experiment

Of the three kids on the calcium supplemented diet which died within five months of the start of the experiment, two had some degree of enteritis throughout the small intestine but no acid-fast organisms were found in smears. The intestines of the third kid were normal. The surviving animal of this group, D. 1896, (killed 39 weeks after the experiment began, after losing condition for some weeks beforehand) had a very emaciated carcase but lesions were confined to the intestines and mesenteric lymph nodes. There was a moderate catarrhal enteritis throughout most of the small and large intestines but no thickening of the mucosa. Large numbers of clumps of acid-fast organisms were found throughout the whole length of the intestines (Fig. 3). The mesenteric lymph nodes were all enlarged and oedematous, some grossly so. Several nodes contained yellow, gritty material almost entirely confined to the periphery of the node and just below the capsule. The extent of this material varied from a few small areas 1 to 2 mm. in size to larger confluent areas covering much of the surface of the node. Sections showed these to be calcareous degenerative foci and smears from these areas revealed vast numbers of organisms resembling *Mycobact. johnei*. Portions of the calcified glands were ground up and injected into guinea-pigs and a pigeon : when killed they showed no evidence of tuberculosis.

In the calcium deficient group two animals, which died four and five months after the start of the experiment, had catarrhal enteritis

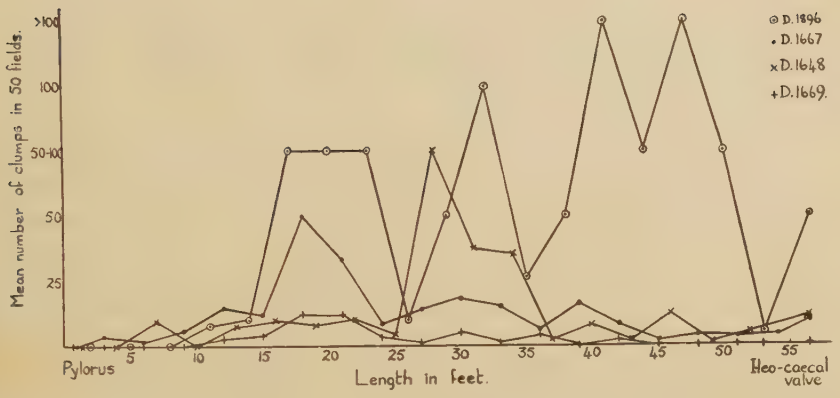

FIG. 3. KIDS ON THE CONTROL DIET.

throughout the small intestine, being very marked in the jejeunum of one, but no acid-fast organisms were found in smears. Only two clumps of acid-fast organisms were found in the intestines (jejeunum) of the other. The third kid, dying after two months, showed a mild catarrhal enteritis and a few acid-fast clumps were found in the duodenum and the ileum near the ileo-caecal valve.

The fourth kid (D. 1940) on the deficient diet was killed in good condition after 41 weeks. Again lesions were confined to the intestines and mesenteric lymph nodes. The small intestine was patchily congested throughout its length with marked inflammation, thickening and corrugation of much of the duodenal mucosa. Only a few clumps of acid-fast organisms were found in the ileum and in the jejeunum. The mesenteric lymph nodes were enlarged and oedematous but did not show any calcified lesions.

Third Experiment

Group on control diet. The carcase of D. 1648, a clinical case, showed marked emaciation, while those of D. 1667 and D. 1669 were in moderate and slightly poor condition respectively.

Intestines. D. 1648 had an enteritis throughout the small intestine with some involvement of the caecum. There was slight thickening throughout the ileum and jejeunum but the lesions were more pronounced with ecchymoses at the distal end of the duodenum. Near the ileo-caecal valve the ileum was thickened and corrugated and the rectum also showed inflammatory thickening of the mucosa. Acid-fast organisms were found throughout the whole of the intestines, being particularly numerous in the jejeunum (Fig. 3). D. 1667 showed slight catarrhal inflammation and thickening of the mucosa in the ileum and jejeunum and some congestion of the duodenal and caecal mucosae. Acid-fast organisms were found throughout the intestine again being more numerous in the jejeu-

num. In D. 1669, most of the duodenum was affected with thickened mucosa and catarrh, gradually decreasing in the jejeunum : a patch of four feet of the ileum was again thickened and inflamed. Small numbers of acid-fast organisms were found throughout most of both large and small intestines.

Lymph nodes. The mesenteric lymph nodes were very swollen and oedematous in all three kids. Calcified lesions, similar to those seen in D. 1896 of the second experiment, appeared in the mesenterics of each : in addition, D. 1648 showed a lesion in the node draining the ileo-caecal valve, while in the other two several nodes were involved. In D. 1669 this type of lesion also occurred in the hepatic lymph node. Lymph nodes other than the mesenterics were sometimes slightly enlarged, the hepatic nodes being most often affected.

Group on deficient diet. All three kids were in fair condition when killed.

Intestines. D. 1644 showed gross thickening, proliferation and inflammation of the caecum especially around the ileo-caecal valve and these changes extended along the first four feet of colon. The terminal part of the ileum was much less changed but thickening occurred in the jejeunum and there was catarrh but no thickening of the duodenum. A small number of acid-fast organisms were found throughout the large and small intestines. D. 1650 showed slight thickening of the distal end of the jejeunum; otherwise the intestine was normal. Large numbers of acid-fast organisms were found, especially in the jejeunum. D. 1665 had some slight increase in mucosal thickness of the proximal end of the ileum, and there were large numbers of acid-fast organisms throughout the intestine (Fig. 4).

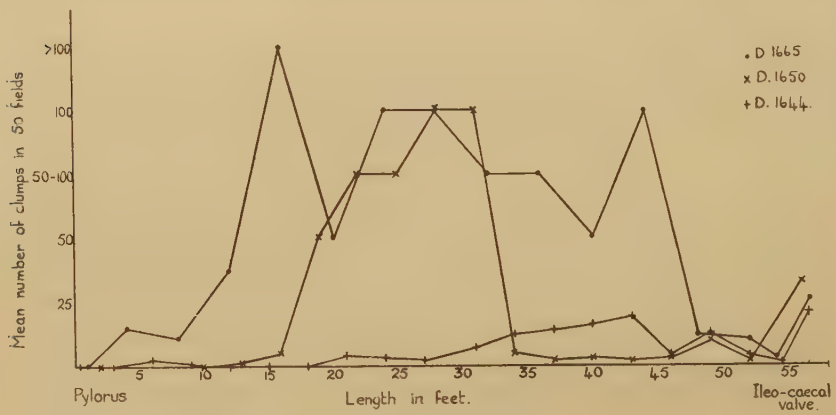

FIG. 4. KIDS ON THE LOW CALCIUM DIET.

The number of clumps in the intestine of D. 1940 was too low to include in the graph.

Lymph nodes. No calcified lesions were seen in any lymph nodes but in D. 1665 most of the mesenteric nodes were considerably enlarged and somewhat fibrous, especially those near the ileo-caecal valve. Numerous hard, white nodules up to 4 mm. in diameter appeared along the course of several lymphatic vessels draining the small intestines, but no acid-fast organisms were found. The hepatic nodes of this kid were also enlarged and oedematous. D. 1644 had gross enlargement of the mesenterics and hepatics, while in D. 1650, whose mesenterics were very big, the hepatic, internal iliac and mediastinal lymph nodes were very obvious.

Cultures

In some respects the results of the cultural tests were unsatisfactory. The egg medium seemed to vary slightly from batch to batch and trouble was experienced with fat on the surface of some of the slopes : this may have inhibited, or slowed down, growth, as well as making it very difficult to see the colonies when present in small numbers. In some cases, even after four months incubation, it was necessary to film the slopes to confirm the presence of colonies. In spite of the oxalic acid treatment, some of the slopes had to be discarded because of the growth of moulds.

The cultural results for the eight surviving kids are summarised in Table 2. Positive cultures were obtained from most of the material examined but there does not seem to be much difference between the calcium deficient and control kids in this respect. A full cultural examination was made of the organs of one of the kids of the third experiment which died of coccidiosis six weeks after its last infecting dose. Positive cultures were obtained from the kidney, liver, ileo-caecal, hepatic, mediastinal, superior cervical and retro-pharyngeal lymph nodes, and from the tonsil. Large numbers of rather moist, yellowish colonies developed from the liver, hepatic and mediastinal glands. The other kid, which died after seven weeks, gave positive cultures from the liver, spleen and ileo-caecal lymph node; these were the only organs examined.

DISCUSSION

It has been suggested by Smythe (1935, 1950) that dairy cattle grazing on land with a soil rich in calcium, are less prone to become clinical cases of Johne's disease than those on farms whose soil is deficient in calcium. He refers to an area on the North coast of Cornwall, with a soil calcium content of 25 per cent, where clinical Johne's disease is very rare—in spite of the presence of infected animals demonstrated at post-mortem. The transference of cows from this region to adjoining granite country, where the soil is acid and the lime content low, frequently leads to clinical breakdown, often within a few weeks. Cunningham, opening the discussion on a paper by Taylor (1952), refers to observations made in New Zealand

TABLE 2

CULTURAL RESULTS

Kid	Time between first infecting dose and death (Weeks)	Group	Liver		Lung		Kidney		Spleen		Tonsil		Lymph nodes: Mesenteric		Hepatic		Bronchial		Retro-pharyngeal		Sub. maxillary		Superior cervical	
			+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—
Second	*Experiment*																							
D. 1896	39	C	*4	0	3	0	4	0	4	0	4	0	4	0	5	0	2	2	4	1	4	1	3	2
D. 1940	41	D	3	0	0	3	—		—		1	3	3	2	—		2	3	2	3	1	4	3	2
Third	*Experiment*																							
D. 1665	28	D	†2	1	2	1	0	3	0	3	2	1	3	0	1	2	2	1	1	2	—		—	
D. 1667	29	C	1	2	0	2	3	0	2	1	—		3	0	1	1	0	2	1	1	0	3	—	
D. 1648	36½	C	0	3	0	3	0	3	—		—		1	2	0	1	1	2	0	2	1	2	1	2
D. 1650	38	D	3	0	0	3	2	1	3	0	3	0	3	0	3	0	3	0	2	0	2	1	—	
D. 1644	39	D	2	1	2	1	1	1	0	3	0	3	3	0	0	3	0	3	3	0	1	2	—	
D. 1669	40	C	0	3	2	1	1	2	1	2	3	0	3	0	2	1	1	2	3	0	2	1	—	

C=Animal in control group.
D=Animal in deficient group.
+=Some growth.
—=No growth.

† The deposit was sown on to three slopes; as grossly contaminated tubes have been omitted from the table, the total of the two columns is not always the same.

* Five slopes were used in the second experiment.

which indicate that soil type may have some bearing on the incidence of Johne's disease. In some districts of the North Island this disease is unknown, although animals from a region of a different soil type, where the disease is common, have been transferred there. Spicer (1933) quotes several instances, where the feeding of a mineral mixture (largely calcium) to cows in herds where Johne's disease was a problem, has been followed by at least two years freedom from new cases. On the other hand, Smythe (1950) mentions two farms whose soil was deficient in calcium, but whose losses from Johne's disease were lower than those on an adjoining farm which had been "limed and overlimed". So far as is known there are no records of laboratory trials to determine the effect of mineral deficiencies on the course of the experimentally induced disease.

In the experiments reported in this paper the serum calcium levels of kids was depressed by a ration similar to that used by Glock, *et al.* (1939), and the figures obtained agree reasonably closely with theirs. In addition, clinical evidence of a calcium deficiency was noted in the production of a frank osteo-dystrophy, an indication that the bone reserves of calcium were being rapidly depleted.

The effect of a low calcium diet in Johne's disease, is, however, complicated by the possibility that in the later stages the disease process itself may induce a mineral imbalance. It is of interest, therefore, that in the third experiment, for which complete data are available, the serum calcium levels of the three controls dropped gradually in spite of the feeding of a calcium supplement. One became a clinical case; and a few weeks before it was killed successive estimations were 7·2 and 7·4 mg. respectively. Unfortunately no estimations of serum calcium were made on the kid, (D. 1896) in the second experiment, after it became a clinical case. The serum calcium values in these animals are similar to those obtained by Stewart, McCallum and Taylor (1945) in Johne's disease in sheep and cattle. The reason for the fall is not known but it may be related to some interference with the absorption of calcium from the intestine.

In both the deficient and control groups there were marked fluctuations in the inorganic phosphorus levels which tended to become abnormally high in the former. This was to be expected, as high phosphorus levels are generally encountered when the blood calcium levels approach the threshold of calcium tetany. Stewart, *et al.* (1945), did not find any appreciable difference from the mean in their blood inorganic phosphorus determinations.

The number of animals subjected to detailed post-mortem examination was too small to give a precise answer to the effects of a calcium deficiency on the course of the disease. The fact that both clinical cases were encountered in the control group would imply that a deficiency of calcium does not accelerate the progress

of infection. In both groups positive cultures were obtained from most of the organs examined; this is of interest but is not unexpected since, in six out of eight kids, the intravenous route of infection had been employed. The recovery of the *Mycobact. johnei* from such sites as the liver and spleen, hepatic and mediastinal lymph nodes, agrees with the findings of Levi (1948). There was a considerable individual variation in the numbers of clumps of acid-fast organisms in intestinal smears and, as is the case in cattle and sheep, no apparent relation between the bacillary population and the severity of the lesions. In general, more bacilli were found in the jejeunum than in the region of the ileo-caecal valve, but this again might have been related to the double route of infection.

A description of the detailed histological findings will be published in another paper. In general no significant difference could be detected in the control and deficient groups except in the mesenteric glands. Although the number of animals available was small, four goats in the control group showed calcified lesions whereas none were found in the group on the low calcium diet. The lesions were mainly subcapsular and were not apparently related to the route of infection. M'Fadyean and Sheather (1916), described similar lesions in natural caprine cases and in goats infected intravenously. They described them as "dry, white, caseous centres" within the nodes. Their findings have been confirmed by Levi (1948), who drew attention to the bosselated appearance of such affected nodes. It is tempting to ascribe the lack of calcified lesions in the group on the calcium deficient diet to the diet which was fed.

CONCLUSIONS

Experimentally induced Johne's disease was studied in kids maintained on a diet deficient in calcium and in a control group receiving the same diet but supplemented with calcium and cod liver oil.

Two out of the eight kids became clinical cases; both were in the control group.

Of the six animals killed before the onset of clinical symptoms, post-mortem examination—including cultures, and examination of intestinal films for clumps of acid-fast organisms—did not demonstrate any obvious differences between the two groups. Attention is drawn, however, to the presence of calcified lesions in the mesenteric lymph nodes of the control animals, and their absence from the animals on the low calcium diet.

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VIABILITY AND IMMUNIZING POTENCY OF FREEZE-DRIED BOVINE CONTAGIOUS PLEUROPNEUMONIA CULTURE-VACCINE

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INTRODUCTION

In 1949, the necessity arose on several occasions to air-freight batches of living bovine contagious pleuropneumonia (BCPP) culture-vaccine from Australia to Ethiopia as part of the Foreign Aid Programme of the Food and Agriculture Organization of the United Nations. The experience of one of us (D.F.G.) immediately prior to this period had been that BCPP was second in importance only to rinderpest in that country. Laboratory facilities in Addis Abbaba and available personnel were not at that time conducive to routine production of the vaccine, but limited field trials were carried out with vaccine prepared as described by Turner, Campbell and Dick (1935) from the spontaneously attenuated V5 strain of *Asterococcus mycoides* (Campbell, 1938a). These trials demonstrated that it was well tolerated by the various types of native cattle and that it gave a satisfactory serological response; however, the period of observation was too brief to permit reliable assessment of immunity. Subsequently, two consignments of vaccine, and one of the dried product described herein, were sent from Melbourne and the field reports following its use during outbreaks of BCPP were satisfactory.

The incidence of BCPP in Ethiopia may be very irregular, resulting in sudden demands for vaccination on a large scale, followed by periods during which no further demands arise. To meet such emergencies, it becomes necessary to hold large stocks of living vaccine for which the usable period is only two months. It therefore seemed desirable to prepare the vaccine in a more stable form. Freeze-drying appeared to offer the most hopeful solution to the problem of ensuring that the vaccine would maintain its potency without refrigeration during the air transport and the unpredictable delays in tropical countries, and that it would reach its destination in a state suitable for further storage for prolonged periods.

After some preliminary trials, a batch of vaccine was lyophilized in January 1950. Some ampoules were held at room temperature; others at 4°C. Viable counts were performed before drying, and at intervals thereafter, and in April 1952, after a period of 27 months, an immunity trial was carried out.

MATERIAL AND METHODS

The vaccine. The main experiment was carried out with a standard culture-vaccine, Batch No. 423. The culture had been incubated at 37°C. for 4 days, after which its " decimal titre " was 10^9. After being stored at room temperature for 10 days, when the titre was still 10^9, samples were lyophilized.

Freeze-drying technique. In the preliminary trials, drying was carried out in the small " dryvac " apparatus (Gray, 1948) used for routine drying of cultures, but, in addition to the usual silica gel desiccant, a container of P_2O_5 was introduced into the vacuum line to cope with the larger volume. Ampoules with a 5 ml. capacity were placed on an Oertling air-damped balance and 1 g. of fluid vaccine added. The filled ampoules were quickly shell-frozen in dry-ice and alcohol, in which they were held until fitted to the " dryvac " apparatus. This was carried out individually and a fresh ampoule was not fitted until the Vacustat reading had returned to 100 μ. The pump was run continuously and any ampoules showing a tendency in the early stages to thaw were re-frozen by immersion in freezing mixture.

The period of drying was varied in the experimental stages from 24 to 120 hours in order to determine the desiccation rate. Ampoules were removed from the apparatus for weight-loss estimations by releasing the vacuum for the minimal necessary period. Finally, they were sealed at a pressure of not more than 100 μ with a crossfire torch.

In the final experiment with batch No. 423 the facilities of the Commonwealth Serum Laboratories, Parkville, were generously made available. Quantities of 5 ml. and 10 ml. were dispensed in 12 ml. and 33 ml. capacity ampoules respectively; they were then shell-frozen, dried for 26 hours and sealed at 100μ. The efficiency of sealing was checked, both then and immediately before opening an ampoule, by obtaining internal fluorescence with an Edwards Betoray No. 1 vacuum tester. Equal numbers of ampoules were stored in the dark at room temperature (climatic range 10°–40°C.) and at 4°C.

Desiccation rate. This was determined by weighing ampoules at 24-hour intervals until a stable reading was obtained.

Titration of vaccine. For routine purposes, " decimal titration " was employed: ten-fold serial dilutions (1 ml. to 9 ml.) were made in BVF–OS culture medium (Turner, Campbell and Dick, 1935) and the tubes were incubated until an end-point was reached, usually by the sixth day. The viable count was then calculated from the highest dilution in which growth occurred. A titre of 10^9 viable cells per ml. (usually abbreviated to " 10^9 ") implies that the end point of growth was the " 10^{-10} " tube of the series, and that 1 ml. of the 10^9 dilution therefore contains not less than one viable unit. Since only single tubes were sown in these series,

the count determined in this way is not very precise. For more precise estimates, the dilution method described by Fisher and Yates (1943) was applied, by preparing within the expected range a series of two-fold dilutions, from each of which five 1 ml. amounts were sown. Sometimes, this was supplemented by direct counts by plating on the surface of blood agar plates which were sealed to prevent evaporation.

Vaccination procedure. The contents of two large ampoules which had been stored at room temperature for 27 months were each reconstituted in 10ml. of sterile distilled water. Half the pooled contents was placed on ice for subsequent titration at the laboratory and the rest was used to vaccinate thirty healthy adult cattle with no history of previous contact with the disease. Record syringes of 1 ml. capacity, fitted with three-quarter inch 18-gauge needles were used. The dose (0·2 ml.) was introduced deeply into the tissues from 3 to 5 cm. from the tip of the tail, virtually axially, the needle being inserted in a cranial direction.

Number of viable organisms inoculated. Titration of the ice-chilled reconstituted vaccine was carried out within three hours at the laboratory. The decimal dilution method gave an estimate of 10^6 viable units per ml. The method of Fisher and Yates (1943) gave an estimated value of $5{\cdot}82 \times 10^6$ viable units per ml., with upper limit $15{\cdot}0 \times 10^6$ and lower limit $2{\cdot}61 \times 10^6$, at the five per cent, level of significance. Direct plating in triplicate gave an estimate of $4{\cdot}29 \times 10^6$ viable units per ml., with limits ($P = 0{\cdot}05$) of $5{\cdot}85 \times 10^6$ and $2{\cdot}72 \times 10^6$. It is therefore likely that the 0·2 ml. dose contained about 10^6 units capable of germinating in the protected environment of culture media.

Serological control. The serological response after vaccination and challenge was determined at intervals by the complement fixation test (CFT) of Campbell and Turner (1953).

Immunity Challenge. This was carried out by exposing animals to nebulized virulent culture according to the method described by Campbell (1938b). The challenging strain was a second subculture from field material from Queensland and was used as a three-day culture in BVF-OS, estimated to contain $3{\cdot}62 \times 10^8$ viable units per ml., of which 600 ml. was nebulized into a sealed stall of capacity $41 m^3$. The animals were exposed in batches of six vaccinated and three control animals for 15 minutes. Clinical observation and serological tests were carried out over the ensuing six weeks: then all surviving animals were slaughtered and the thoracic organs were subjected to pathological and bacteriological examination.

Criteria of susceptibility and resistance. Since the animals were sheltered, well fed and kept at rest, clinical signs of disease tended to be suppressed. Furthermore, non-specific thermal disturbances occurred because of advanced pregnancy or of chronic fascioliasis. Hence, only minor significance is attached to the clinical evidence. The conclusions are based upon the pathological findings, and particularly the bacteriological findings, at autopsy six weeks after exposure to nebulized culture. Recovery of *A. mycoides* from lesions or from mediastinal or bronchial lymphatic glands (Campbell and Turner, 1936), is taken as evidence that the animal was susceptible, in that it was not able to rid itself of infection within six weeks. This was virtually always associated with the necrotic sequestrated lesions or with resolving lesions. Absence of lesions, or the presence of slight adhesions, usually with some local fibrotic thickening of the

pleurae, as the only abnormalities, was virtually always associated with failure to demonstrate *A. mycoides* in the thoracic lymphatic glands; this is taken as evidence that the animal was naturally resistant or successfully immunized, since the infection did not lead to significant disease, but merely to small local lesions which had healed without important vascular or lymphatic thrombosis.

RESULTS

Effect of Lyophilization upon the Vaccine

Desiccation Rate

Several ampoules containing 1 g. of vaccine were shell-frozen and attached to the "dry-vac" apparatus. Drying proceeded at a negative pressure of approximately 100 to 150μ. The average weight loss was as follows :—24 hours, 93·35 per cent; 48 hours, 93·67 per cent; 72 hours, 93·74 per cent; 96 hours, 93·74 per cent; 120 hours, 93·74 per cent. Thus, with the "dryvac" apparatus, most of the available moisture had been removed at 24 hours, but further small amounts were given up until stability was reached between the 48th and 72nd hours.

Immediate Lethal Effect

The decimal titre before lyophilization was 10^9. After 24 hours, reconstituted vaccine had a titre of 10^7. This was maintained at 48, 72 and 96 hours, indicating that under these conditions an approximately 99 per cent mortality occurred during the initial drying period, after which survivors became relatively stable at about 1 per cent of the original population.

Influence of Storage upon Viability

In these tests, the 5 ml. and 10 ml. quantities dried for 24 hours in 12 ml. and 33 ml. ampoules, as finally used for the immunization challenge, were used.

Storage at room temperature. Decimal titrations were performed before drying and then at intervals of approximately one month for the first 8 months, followed by less regular intervals up to 41 months. The initial titre of 10^9 dropped to 10^7 during drying, and this value was maintained each month for 8 months, except for two counts of 10^8 at the third and fifth months, probably referable to the inherent sampling errors of the technique. After 14 months the titre was 10^6 but was again 10^7 on two later occasions. The titre of the ampoules opened after 27 months for immunization was 10^6 and after 41 months it was again 10^6. Because of the low precision of decimal titration estimates, it is not certain from these tests that the titre of lyophilized vaccine had decreased significantly during storage for 41 months at room temperature, but there appears to be a trend towards lower viability after 14 months. Titration by the more

precise method of Fisher and Yates was carried out after storage for 27 months and 41 months, and revealed a slight decrease in viability (Table I).

TABLE I

INFLUENCE OF STORAGE TEMPERATURE UPON VIABILITY OF LYOPHILIZED PLEUROPNEUMONIA VACCINE

Interval after lyophilization (months)	*Storage temperature*	*Titre* (x 10^6/ml.)			*Ratio of means (4°/room temp.)*
		Max. (P=0. 025)	*Estimated*	*Min.* (P=0. 025)	
27	Room	15·0	5·82	2·61	
32	4°C.	34·6	18·8	10·36	
41	Room	5·09	3·10	1·69	4·9
41	4°C.	28·0	15·2	8·29	

Storage at 4°C. Titration by the method of Fisher and Yates was carried out on ampoules stored at 4°C. for 32 and 41 months respectively. The results, given in Table I, indicate a general trend towards decreasing viability as time progresses, at each storage temperature, and also demonstrate that the viability of ampoules maintained at 4°C. was greater by a factor of about 5 than that of ampoules maintained at room temperature for 41 months.

Immunizing Potency of Lyophilized Vaccine

Response to Vaccination

The relationship between tail reactions, CF response and subsequent resistance to challenge is shown in Table II.

Tail reactions. In general, the tail reactions were relatively slight and delayed. Only 6 animals developed readily detectable swellings at the tip of the tail. Of these, 3 (14, 204 and 225) lost a small amount of necrotic tissue from the site of inoculation by sloughing, and another (311) lost the terminal 5 inches of the tail; in the other (258) an oedematous inflammatory swelling, involving the terminal 3 to 4 inches, persisted during the five weekly inspections. Material from 3 out of the 4 tails in which sloughing occurred yielded *A. mycoides* when sown into selective medium.

Three other animals developed very transient slight oedematous reactions, without sloughing. Thus 21 out of 30 animals (70 per cent) failed to develop recognizable local reactions.

Serological response. Nine animals (30 per cent) reacted strongly to the CFT (i.e., titre >20), four (13·3 per cent) reacted moderately (i.e., titre = 10 to 20) and 17 (56·7 per cent) failed to react (i.e., titre <10).

TABLE II

IMMUNIZING POTENCY OF LYOPHILIZED PLEUROPNEUMONIA VACCINE

Vaccinated Group (30)													*Unvaccinated Group* (15)							
	Response to vaccination				*Response to challenge*									*Response to challenge*						
	Tail reactions		*Max. CFR*		*Max. CFR*		*Lesion*			*Culture*				*Max. CFR*		*Lesions*			*Culture*		
No.	*Interval (days)*	*Extent (cm.)*	*Titre*	*Interval (days)*	*Titre*	*Interval (days)*	A	R	S	L	G	*Interpretation*	*No.*	*Titre*	*Interval (days)*	A	R	S	L	G	*Interpretation*
9	—	—	—	—	20	43	+	+	—	+	+	Susc.	51	40	18	—	—	—	O	+	Susc.
10	—	—	—	—	20	8	+	—	—	O	—	Res.	80	80	18	—	—	+	C	C	Susc.
12	—	—	—	—	20	18	+	—	—	O	—	Res.	91	160	22	+	+	+	+	+	Susc.
13	—	—	—	—	80	37	—	+	—	C	+	Susc.	113	40	22	+	+	—	+	+	Susc.
14	42	1	80	28	0	0	—	—	—	—	—	Res.	145	160	18	—	—	+	+	—	Susc.
15	—	—	10	35	40	25	—	—	—	O	—	Res.	168	80	16	+	—	+	+	+	Susc.
21	—	—	—	—	10	8	+	—	—	O	—	Res.	171	160	16	—	+	+	+	—	Susc.
22	35	<1	80	28	40	8	—	—	—	O	—	Res.	192*	320	18						Susc.
82	—	—	—	—	160	25	+	—	—	O	—	Res.	246	640	32	+	+	+	—	+	Susc.
148	—	—	—	—	160	32	+	+	—	+	—	Susc.	266	160	16	+	+	+	+	—	Susc.
156	—	—	—	—	80	18	+	—	—	O	—	Res.	274	80	32	+	—	+	C	+	Susc.
200	—	—	—	—	640	8	—	—	—	O	—	Res.	290	320	16	—	+	+	+	C	Susc.
203	—	—	160	21	160	11	—	—	—	O	—	Res.	335	40	18	—	—	—	O	—	Res.
204	42	1	20	35	160	8	—	—	—	O	—	Res.	363	320	32	—	+	+	+	+	Susc.
228	35	<1	—	—	80	18	—	—	—	O	—	Res.	367	320	25	—	—	+	+	—	Susc.
244	—	—	20	35	40	25	—	—	—	O	—	Res.									
248	—	—	160	14	320	8	+	—	—	O	—	Res.									
256	—	—	80	21	320	18	+	—	—	O	—	Res.									
258	28	9	80	14	160	8	+	—	—	O	—	Res.									
275	35	1	40	14	160	16	+	—	—	O	—	Res.									
295	21	<1	—	—	80	32	—	—	—	O	—	Res.									
296	—	—	—	—	10	16	—	—	—	O	—	Res.									
305	—	—	—	—	40	43	—	—	+	+	+	Susc.									
311	42	13	40	35	320	8	+	—	—	O	—	Res.									
323	49	9	20	35	20	8	—	—	—	O	—	Res.									
324	—	—	—	—	20	8	—	—	—	O	—	Res.									
342	—	—	40	49	160	8	—	—	—	O	—	Res.									
346	—	—	—	—	20	11	—	—	—	—	—	Res.									
360	—	—	—	—	20	8	+	—	—	—	+	Susc.									
368	—	—	—	—	80	25	—	—	+	+	—	Susc.									

Abbreviations: A= fibrous adhesions; C=contaminated cultures, therefore not possible to decide if *A.mycoides* present; G=mediastinal or bronchial lymphatic glands; L=lesions; O=no pulmonary lesion, therefore culture not attempted; R=resolving lesions; Res.=resistant; S=sequestrated lesions; Susc.=susceptible.

Response to Immunity Challenge

Five months after vaccination, the resistance of the 30 vaccinated animals and of 15 unvaccinated controls of similar origin and age was challenged as described. After clinical and serological observations for 6 weeks, 44 were submitted to pathological and bacteriological examination. During this period, control animal 192 had become gravely ill with acute pleuro-pneumonia and had been killed after 21 days. Three other control animals (246, 274 and 290) and two vaccinated animals (200 and 368) had developed relatively severe signs of illness, comprising coughing, loss of condition and hyperthermia for a week or more, but no deaths occurred.

A summary of the findings is included in Table II, from which it is seen that, judged by the criteria described, 24 out of 30 (80 per cent) of the vaccinated animals were protected against a challenge that produced significant lesions and persisting infection in 14 out of 15 (93·4 per cent) of unvaccinated control animals. For these findings, $\chi_c^2 = 18{\cdot}9$, which for 1 d.f., leads to $P < 0{\cdot}001$.

We have accepted the presence of fibrotic adhesions 6 weeks after exposure to infection, and the absence of *A. mycoides* from regional lymphatic glands, as compatible with effective immunity, and indeed as indicative of it ; however, the findings can also be examined on the basis of complete absence of macroscopic lesions attributable to abortive pleuropneumonia. Judged by this extreme criterion, 14 out of 30 (46·7 per cent) vaccinated animals were apparently completely protected against challenge which produced specific lesions in 14 out of 15 (93·4 per cent) control animals. For these findings, $\chi_c^2 = 5{\cdot}51$, which, for 1 d.f., leads to $0{\cdot}01 < P < 0{\cdot}02$. Therefore, there can be no doubt that vaccination with reconstituted lyophilized vaccine protected from 47 to 80 per cent of animals, depending upon the criteria of resistance which are adopted.

Relationship of Tail Reactions and CF Reactions to Subsequent Immunity

It is interesting to note that, in 9 (38 per cent) of the 24 animals found to be immunized, neither tail reactions nor CF reactions had been observed after vaccination. All 13 animals which developed CF reactions were resistant to challenge, and 10 out of the 17 (59 per cent) which failed to develop CF reactions were nevertheless resistant. For these findings $\chi_c^2 = 4{\cdot}87$ and $0{\cdot}02 < P < 0{\cdot}05$. It appears, therefore, that an inoculum which does not give rise to local swelling or to CF reactions may yet bring about immunity in many cases ; but immunity is more certain if the inoculum is sufficiently potent to stimulate a positive CF reaction.

DISCUSSION

When dried for 26 hours in the medium in which it was grown *A. mycoides,* like many Gram-negative microorganisms, suffered a high mortality, the viable count being reduced to about 1 per cent

of the original. However, once lyophilized, the vaccine was relatively stable : the viability was maintained at a virtually constant level with only a slight deterioration, for at least 41 months, even at room temperature, and was improved if the product was maintained at refrigerator temperature. Most of the water loss occurred during the first 24 hours of lyophilization, coincident with the hundred-fold fall of viability, and the subsequent very small loss of water during drying for 3 days did not appear to cause a further fall detectable by the decimal-titration method. On the contrary, previous experience (Fry and Greaves, 1951; Gray, to be published) with both Gram-negative and Gram-positive bacilli suggests that it may be undesirable to extract the small quantity of water remaining after drying for 24 hours. For this reason, and because in any case the small "dryvac" apparatus was not capable of drying the large amount of material required, the immunization trial was carried out with vaccine dried by means of the large apparatus for 26 hours before sealing. Although the degree of desiccation was not determined, the fall of viability was of the same order as occurred with the "dryvac" apparatus and it is therefore probable that conditions were not significantly different.

Despite the initial reduction in viable count by about 99 per cent, dried vaccine stored in the dark at room temperature for 27 months was found capable of giving rise to tail reactions and to serological response in a proportion of the animals after vaccination. The tail reactions were less frequent and more delayed in onset, and CF reactions also were less and occurred later than usually follows the use of fresh liquid vaccine (Campbell and Turner, 1953). Nevertheless, 80 per cent of the vaccinated animals, including all which developed CF reactions after vaccination and almost 60 per cent of those that failed to develop CF reactions, were sufficiently immunized to overcome completely the infection brought about by exposing them to a large dose of nebulized culture which produced 93 per cent of persistent infections and significant lesions in unvaccinated animals. Experimental results collected during many years by Campbell (to be published) indicate that about 97 per cent of animals vaccinated with fresh liquid vaccine are immunized, but this information is not based upon the bacteriological criteria used in the present study. However, it may well be that the degree of protection recorded by us after vaccination with lyophilized vaccine is less than that which follows the use of liquid vaccine.

The explanation of these findings is probably related to the relatively smaller number of viable cells (about 10^6) introduced into the tail than when liquid vaccine is used. Freshly prepared liquid vaccine usually contains about 2×10^9 viable cells per ml.; the information on immunizing potency accumulated over many years by Campbell has therefore been based upon intracaudal vaccination with about 4×10^8 viable cells; i.e., about 400 times the number of

viable cells present in 0·2 ml. of the reconstituted lyophilized vaccine. Observations by Dick (1938) have shown that tail reactions are less certain and are less extensive when fresh liquid vaccine is diluted 10^{-2} to 10^{-3}, and that at greater dilutions they may fail altogether.

There is no evidence that the cells in the reconstituted vaccine were less able than undried cells to establish themselves in the tissues although this is, of course, possible; but at least they were able to grow in BVF–OS culture medium without appreciably longer lag period than that of fresh cells : the mean period for the development of recognizable growth of 16 batches of fresh vaccine at limiting dilutions (probably single cells) has been 5 days (range 4 to 7), whereas with the lyophilized vaccine stored either at room temperature or at 4°C. for 27 months and 41 months, the periods after which no further dilution tubes developed growth were 5, 5, 5 and 6 days respectively.

The results of these experiments are considered to be encouraging, but it appears that efforts should be directed towards ensuring that a larger number of viable cells are present in the reconstituted vaccine. It is likely that this may be achieved firstly, by selecting for lyophilization batches of vaccine with the highest possible initial count; secondly by improving the lyophilization process, possibly by replacing the medium in which vaccine was grown by more suitable vehicles, or by the use of protective adjuvants such as glucose (Fry and Greaves, 1951) or lactose (Rosenthal, 1952) or by reducing the degree of desiccation in the absence of glucose (Gray, to be published); and thirdly by storing lyophilized vaccine at a low temperature rather than at room temperature.

A stable dried vaccine of the requisite potency could be especially useful in countries with poorly organized laboratory services, where requirements for the vaccine are unpredictable and where transport difficulties render it necessary to have a supply of vaccine available at short notice. Although the processing, and the provision of extra containers of sterile distilled water in some situations, would increase the cost of the vaccine, this would be off-set by lower freight charges and less wastage. The convenience of having vaccine on hand might also justify greater costs, even in Australia, where air services are greatly developed.

It is interesting to recall that, as early as 1906, Cameron in Victoria suggested the use of dried vaccine and showed that wool "setons" soaked in "natural virus" (i.e. pleuritic exudate or lung juice) and dried at 37°C., could be used successfully for vaccination for at least three months. More recently, Priestley, Miheimid and Dafaalla (1948) and Priestley (1952) have shown that cultures can be dried successfully *in vacuo* over P_2O_5. Their lyophilized cultures were viable for at least three years when stored in the ice chest and for at least sixteen months when stored at 37°C. However, they recorded an increasing lag phase when lyophilized culture was

sown into medium and also formed the opinion that the survivors of dried attenuated cultures were more virulent than the original cultures. There was no suggestion of increased virulence after lyophilization in our experiments; indeed, this would be contrary to experience with all other microorganisms.

SUMMARY

Bovine contagious pleuropneumonia vaccine of Turner, Campbell and Dick (living culture of *A. mycoides* in BVF–OS medium) was lyophilized by freeze-drying. After an initial fall to about 1 per cent of the original number, viable count decreased only slightly during at least 41 months.

After 27 months' storage at room temperature, vaccine was reconstituted with distilled water and used to vaccinate 30 head of cattle intracaudally.

The reconstituted vaccine, which contained in the standard dose about 10^6 viable organisms, gave rise to tail reactions and CF responses that were more delayed, less frequent and less extensive than is usual after the use of fresh liquid vaccine, in which the number of inoculated viable organisms is about 400 times greater. Nevertheless, 80 per cent of the animals were satisfactorily immunized, including 38 per cent of those which failed to develop either local or CF reactions.

It is suggested that the number of viable organisms in the inoculated dose (approximately 10^6) is close to the minimum immunizing threshold and that greater success should be possible if the number of surviving viable organisms could be increased. Possible means of doing this are discussed.

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STUDIES ON TRICHOSTRONGYLOSIS

I. The Pathogenesis of *Trichostrongylus axei* in Sheep maintained on a low Plane of Nutrition

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INTRODUCTION

Parasitic gastro-enteritis of sheep is caused by a group of some six or eight species of trichostrongylid nematode and observations in the field on outbreaks of the disease indicate that these worms are not all equally pathogenic. An exact evaluation of the pathogenesis of these several worms can, however, only be ascertained from laboratory studies on sheep carrying infestations of a single species. Such studies of a number of these worms have been undertaken to some degree by several workers but it is important that more work should be done. One of the worms, *Trichostrongylus axei,* appears to have been overlooked in laboratory studies on pure infestations and so work has been initiated to study its pathogenesis. Since nutrition has been shown to exert a profound effect upon the pathogenesis of parasitic nematodes in sheep a study has first been made of the pathogenesis of *T. axei* in sheep maintained on a low plane of nutrition and this is recorded in this paper. Future papers will record the differences observed when sheep are maintained on a full diet.

MATERIAL AND METHODS

Eight cross-bred down lambs, which had been reared under special conditions designed to keep them free from all trichostrongylid nematodes, were used . It was found impossible, except by adopting a laborious technique, to keep them entirely free from parasites, and they were carrying infestations of *Strongyloides papillosus* and coccidia. As these infestations were of short duration and were present in all lambs used, it is not considered that they could have exerted any influence on the course of the experiment. Of the eight lambs, six, numbers 770, 772, 808, 837, 839 and 854 were experimentally infected with *T.axei,* and the others, numbers 821 and 833, were kept as controls.

The pure culture of *T.axei* used in the experiment was obtained using the horse as a filter in the following manner:— A mixted culture of trichostrongylid larvae (including those of *T.axei*) from the faeces of an infected sheep was fed to a horse in which only the larvae of *T.axei* developed. From its faeces a culture was prepared from which was obtained a mixture of larvae of *T.axei* and horse strongylids. This culture was fed to a worm free lamb, and resulted in the establishment of a pure

infestation of *T.axei*. The purity of the infestation was checked at post-mortem examination after sufficient larvae had been accumulated for purposes of the present experiment.

During the experimental period the lambs were kept on a low plane of nutrition, their diet consisting of hay only. After a preliminary period of one week the six lambs 770, 772, 808, 837, 839 and 854 were dosed daily with a suspension of 4,000 infective larvae until a total of 100,000, had been given to each. Daily inspection was made for evidence of clinical signs of ill health, and in addition on Mondays, Wednesdays and Fridays faecal samples were collected and examined for nematode eggs. A modification of the Clayton-Lane flotation technique was used unless the number of eggs present exceeded 100 per gram, when a modified McMaster technique was employed. Each Monday evening all food and water was removed and not again allowed until the lambs had been weighed the following Tuesday morning. In this way aberrant weight changes caused by the ingestion of large quantities of food or water just before weighing were avoided. On Thursdays a sample of blood was taken for haematological examination. Ten cubic centimetres were drawn into a test-tube, containing one cubic centimetre of 7·5 per cent sodium citrate as an anticoagulant and a further one or two cubic centimetres were collected in a solid watch glass without anticoagulant and used for the preparation of blood films. The films, stained by the May-Grunwald-Giemsa method, were used in making a differential blood count, the examination being carried out by Schillings, four field meander method Two hundred cells were differentiated and classified according to the criteria laid down by Holman (1944) except that " young forms " and " band forms " were classified together as " band forms." The citrated blood was used for the estimation of the total erythrocyte count, total leucocyte count, packed cell volume and haemoglobin content. The erythrocyte count made on blood diluted 200 times using a Thoma pipette was examined in a haematocytometer having Thoma ruling, the cells in 80 small squares being counted. Estimation of the total leucocyte count was made on blood diluted 20 times in a Thoma pipette; the leucocytes in the whole of the ruled area of a haematocytometer with Thoma ruling being counted. Specially calibrated tubes were filled with citrated blood and centrifuged for 80 minutes at 2,000 r.p.m., the packed cell volume being read off as soon as the tubes were removed from the centrifuge. The haemoglobin content was estimated using the Newcomer method. From the results two indices, mean corpuscular volume, and mean corpuscular haemoglobin concentration were calculated.

All lambs were examined post-mortem either when they died during the course of the experiment or after slaughter, and the worm burden of the several parts of the gastro-intestinal tract was estimated using a dilution technique.

RESULTS

The protocols of this experiment are too voluminous for publication but they are kept at the Veterinary Laboratory, Weybridge, where they may be seen by anyone who is interested. For purposes of publication the results have been summarised in the form of eight diagrams, one for each lamb, together with Table I, and the salient points are outlined below.

TABLE 1

POST-MORTEM EXAMINATION OF EXPERIMENTAL LAMBS

Lamb No.	*No. of worms present*			*Chief Lesions*
	Abomasum	*Small Intestine*	*Large Intestine*	
821*	0	0	3 Trichuris ovis	Carcase fair
833*	0	0	5 Trichuris ovis	Carcase fair
772	23,650 T.axei	6 Strongyloides papillosus	2 Trichuris ovis	Carcase, poor, serous atrophy of body fat. Slight inflammation in the abomasum.
808	55,700 T.axei	26 T.axei	7 Trichuris ovis	Carcase poor. Severe gastritis. Fatty degeneration of the liver.
837	51,800 T.axei	3,650 T.axei	4 Trichuris ovis	Emaciated. Serous atrophy of body fat. Slight gastritis.
770	57,200 T.axei	0	2 Trichuris ovis	Carcase fair
839	6,800 T.axei	0	2 Trichuris ovis	Carcase fair
854	17,000 T.axei	200 Strongyloides papillosus	2 Trichuris ovis	Carcase fair

*=Controls.

Although maintained on a low plane of nutrition the control lamb 821 remained almost constant in weight during the experimental period (Fig. 1) whilst 833 gained weight slightly (Fig. 2). The blood pictures of these two sheep showed no abnormal variations and the values showed close correlation with the standards recorded by Holman (1944) for normal sheep, except that the packed cell volume was constantly higher than the value given by Holman. It can be taken, therefore, that these two lambs represent the normal for housed lambs maintained on a low plane of nutrition.

By comparison the following changes were noted in the three experimental infected sheep which died during the course of the investigation.

Lamb 808 began to lose weight rapidly from the time the worms became mature and this tendency continued throughout the rest of the animal's life, the lamb losing 39 pounds in 79 days (Fig. 3). There were, however, no changes in the blood picture.

Lamb 772 began to lose weight soon after infection. Three weeks later, when the first worms reached maturity, the rate of weight loss increased and was rapid until the time of death (Fig. 4). An oligocythaemia began to develop nine weeks after infection and this became progressively more severe until the time of death. A slight neutrophilia also developed, after the tenth week of the experiment.

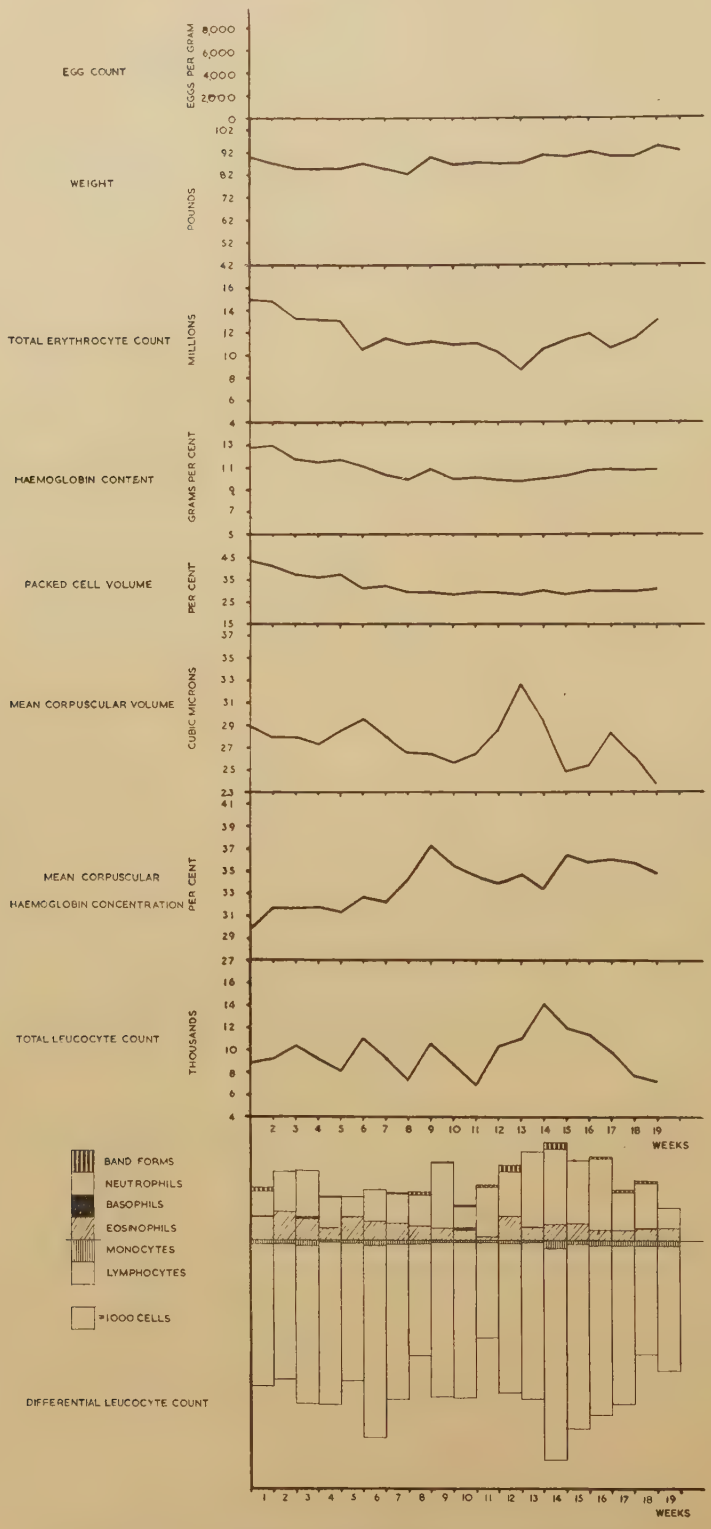

FIG.1 LAMB 821. GRAPHICAL REPRESENTATION OF THE RESULTS.

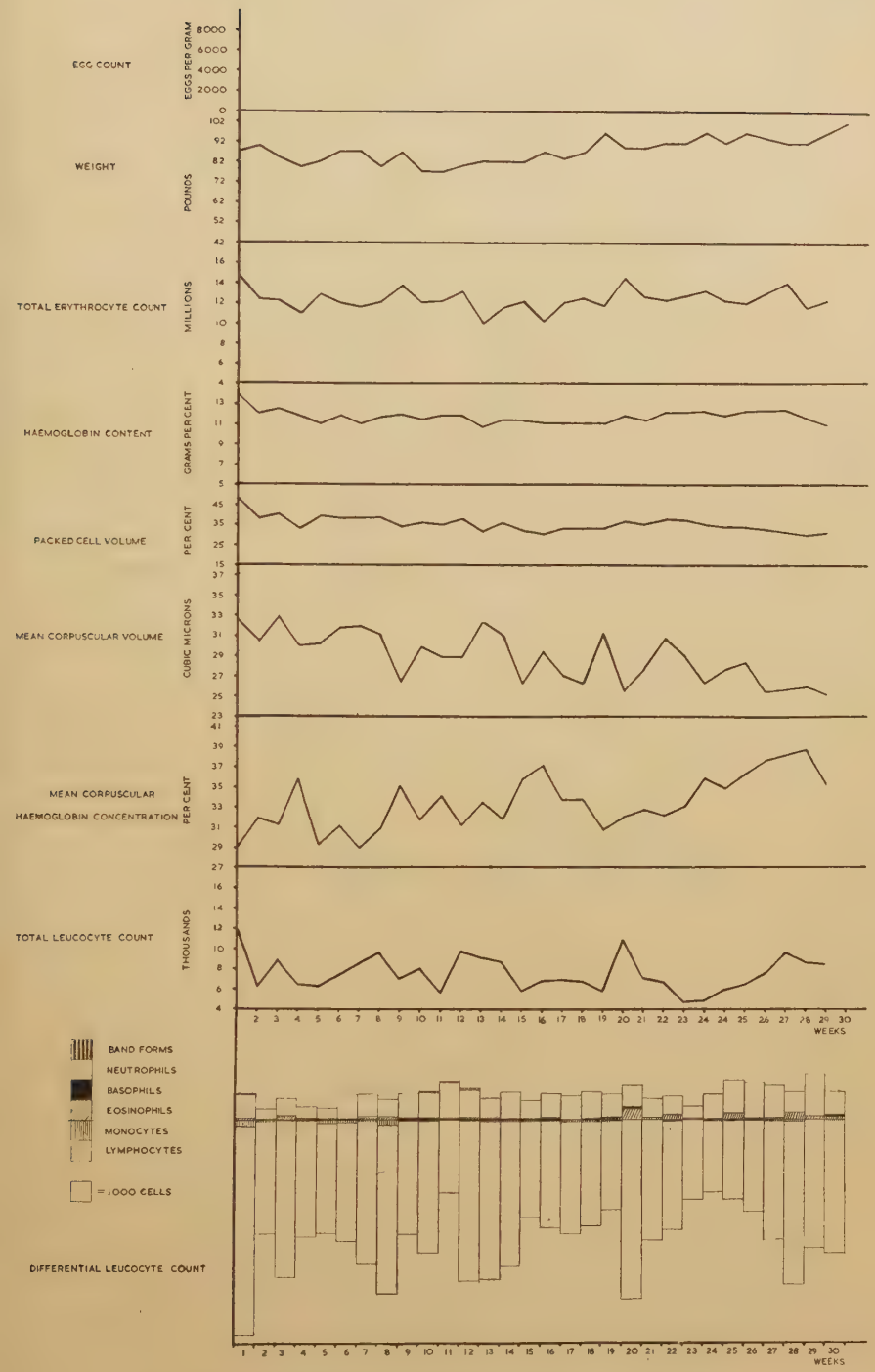

FIG. 2. LAMB 833: GRAPHICAL REPRESENTION OF THE RESULTS.

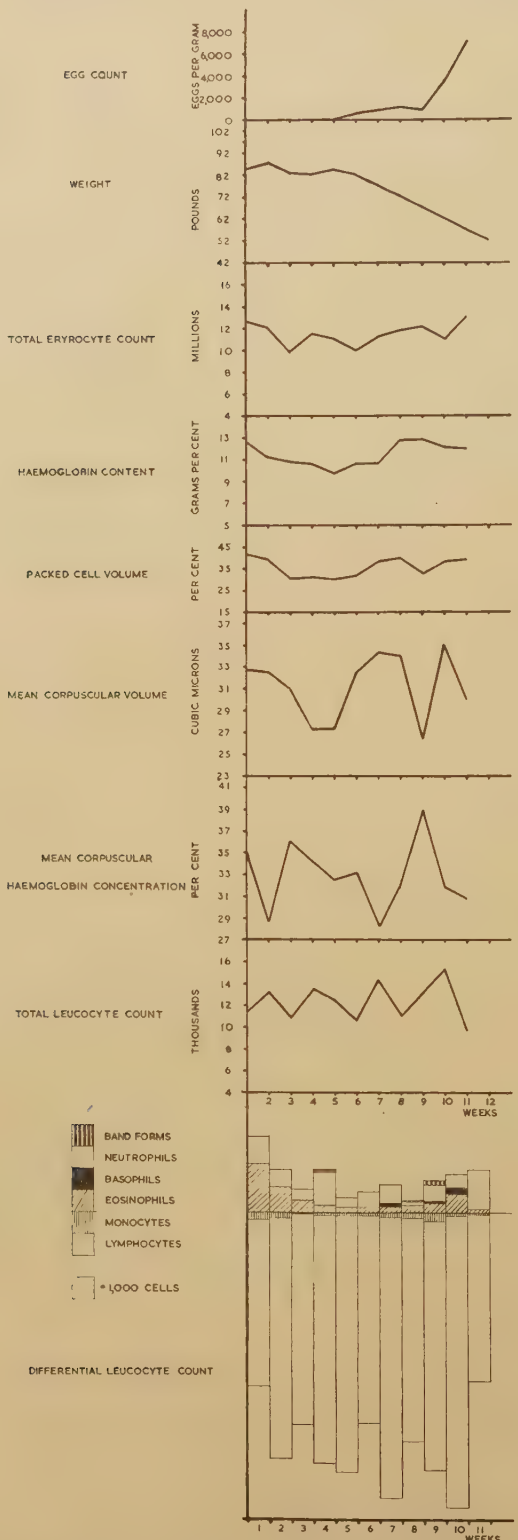

FIG.3 LAMB 808 · GRAPHICAL REPRESENTATION OF THE RESULTS.

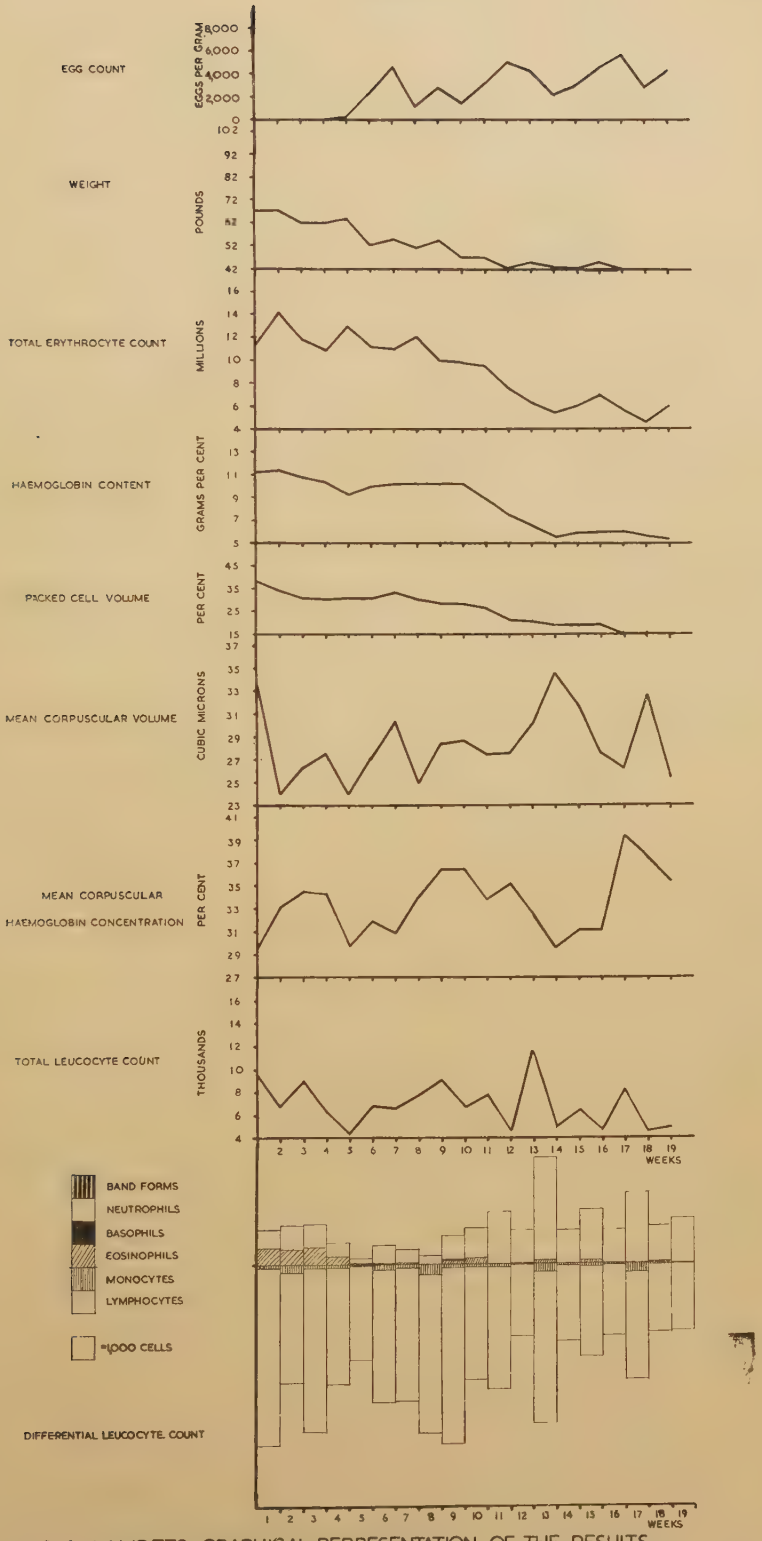

FIG. 4 LAMB 772. GRAPHICAL REPRESENTATION OF THE RESULTS.

D

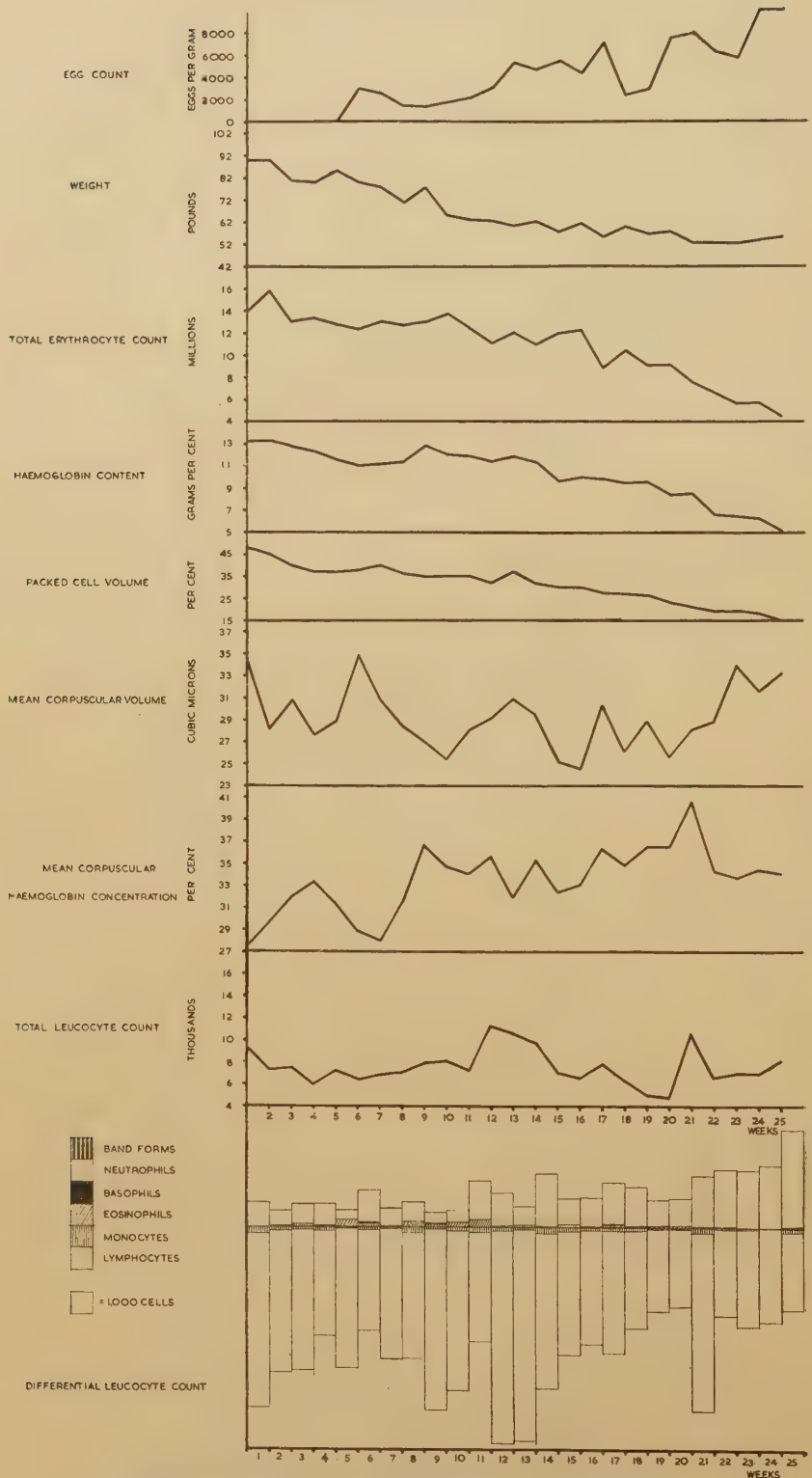

FIG. 5 LAMB 837: GRAPHICAL REPRESENTATION OF THE RESULTS

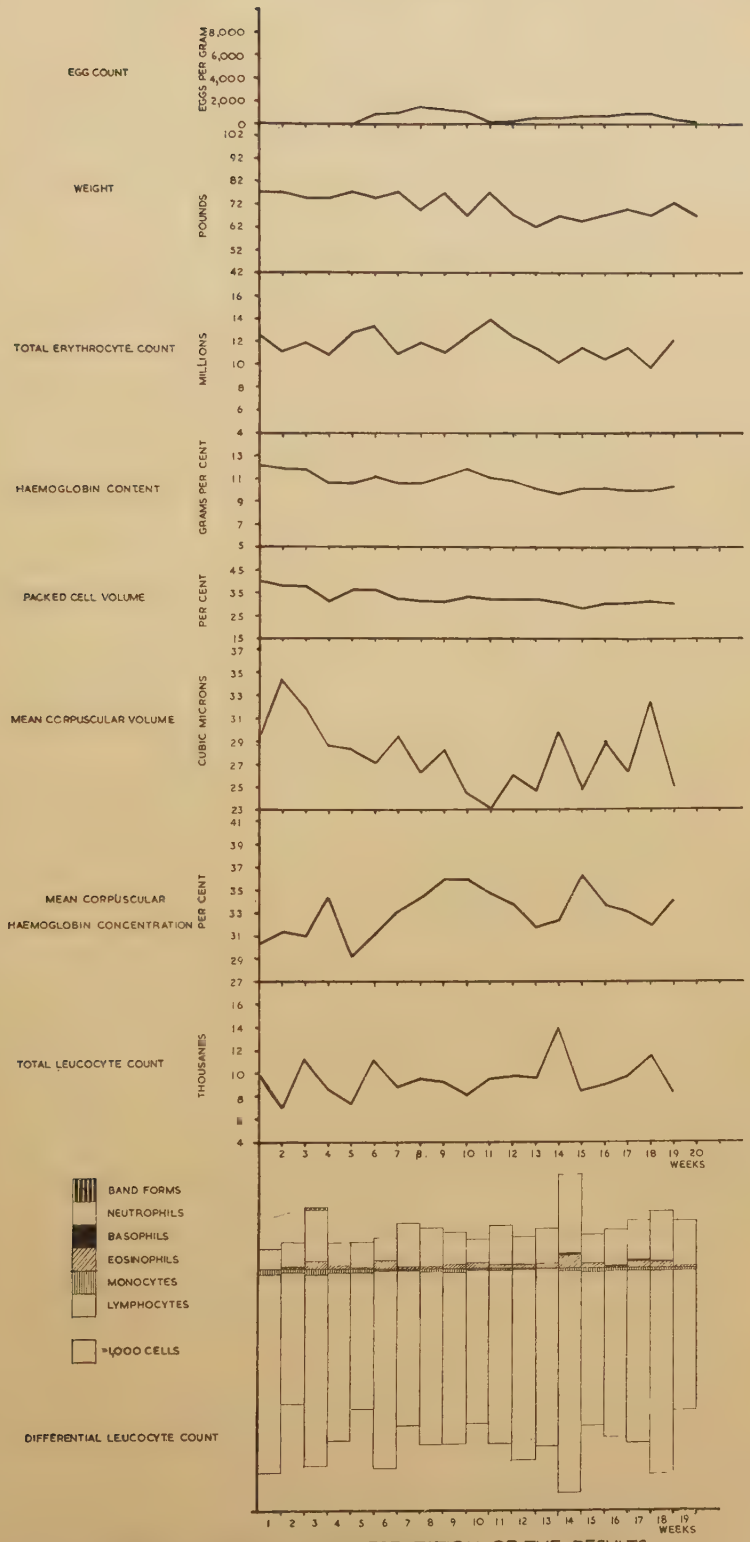

FIG. 6 LAMB 770: GRAPHICAL REPRESENTATION OF THE RESULTS.

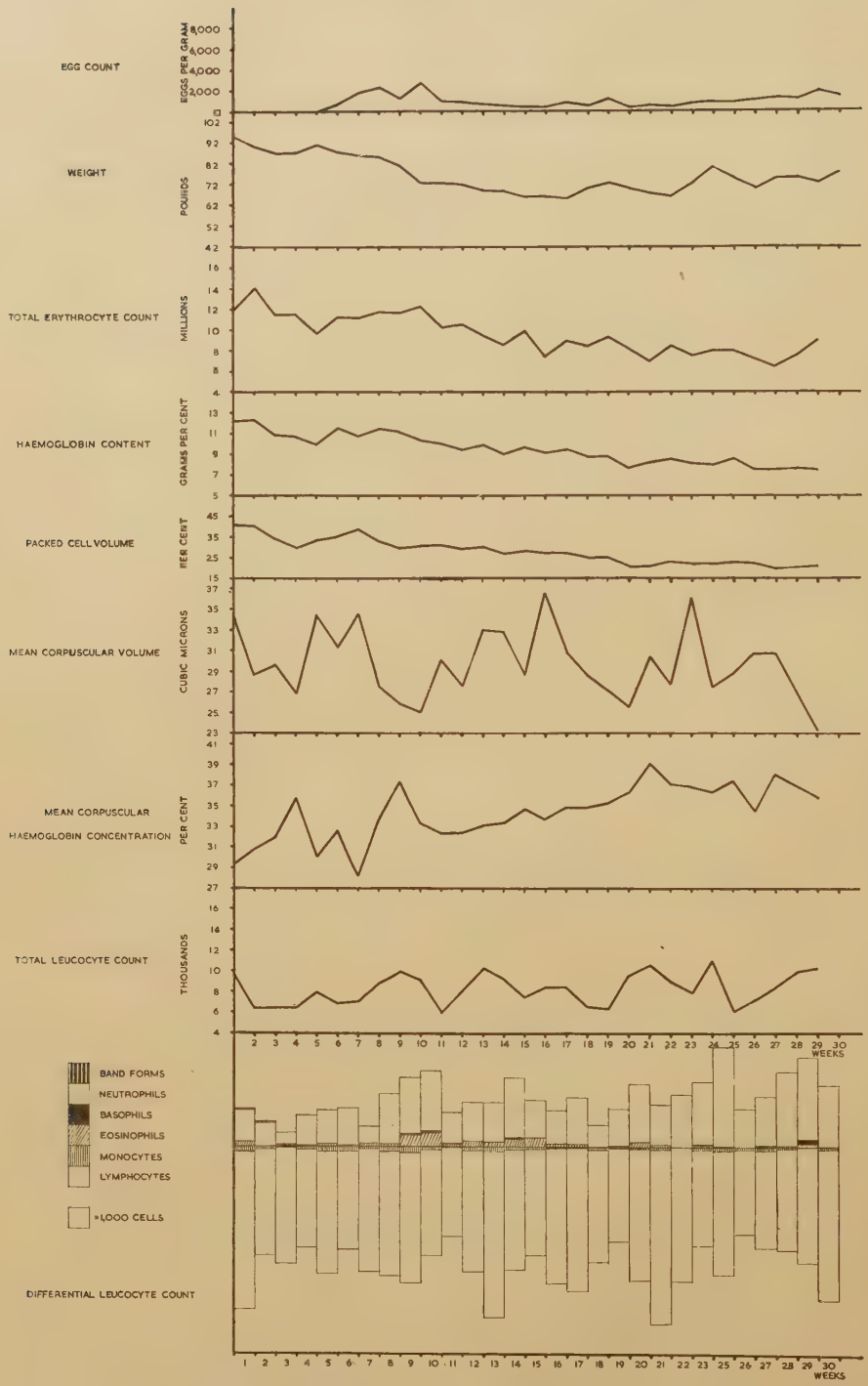

FIG 7 LAMB 839. GRAPHICAL REPRESENTATION OF THE RESULTS.

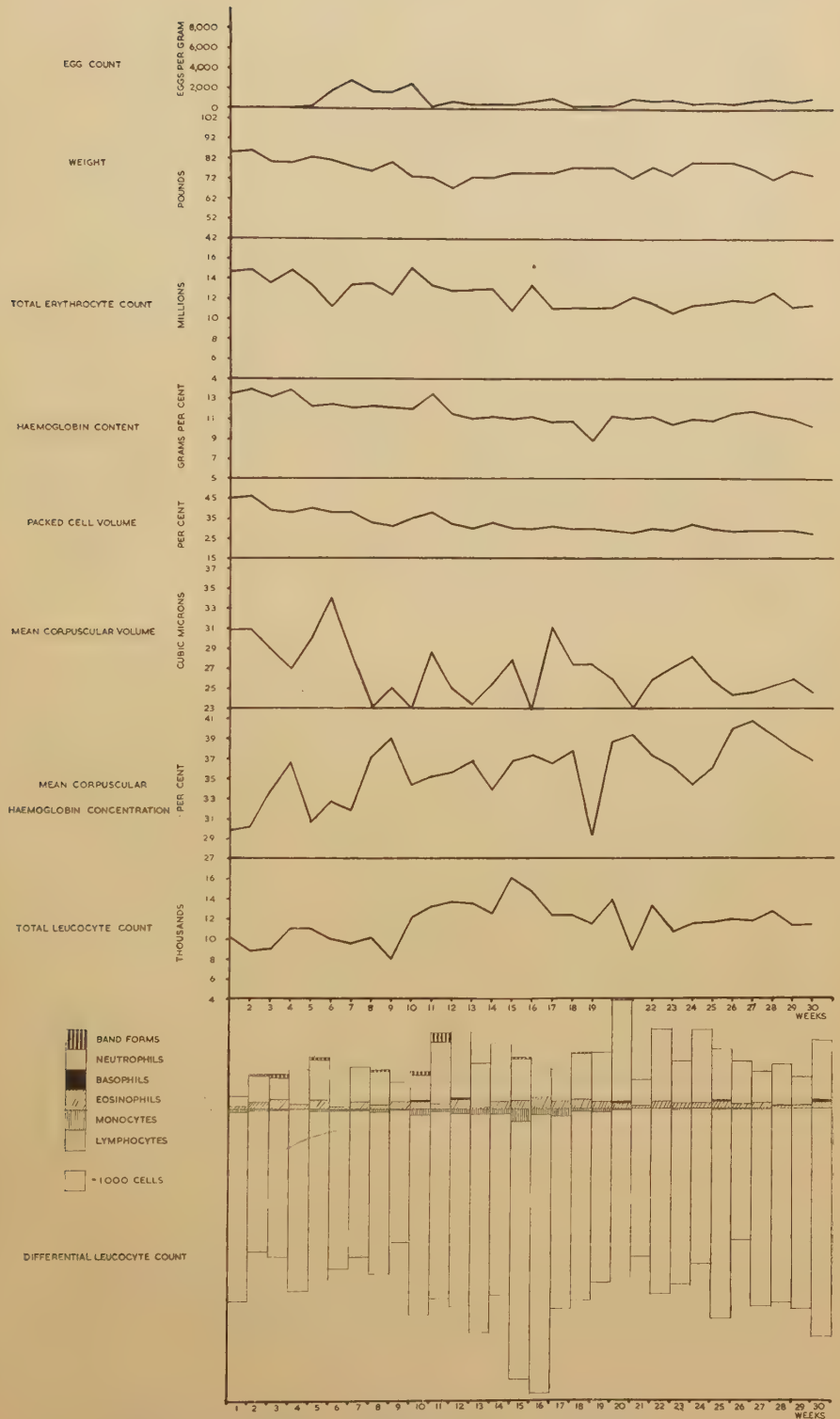

FIG. 8 LAMB 854. GRAPHICAL REPRESENTATION OF THE RESULTS.

The weight of lamb 837 began to fall after the first week of the experiment and this fall continued more rapidly after the worms became mature and continued throughout the rest of the animal's life; the total weight loss over this period amounted to 35 pounds (Fig. 5). Changes in the blood picture did not develop till the tenth week when an oligocythaemia commenced becoming severe by the time the animal died. During the twenty second week this lamb developed a neutrophilia which persisted until the time of its death.

The other three sheep did not die as a result of the infection and did not show such severe effects as have been described above. Lamb 770 lost 10 lb. in weight during the experiment but showed no change in the blood picture (Fig. 6); it was slaughtered in the twentieth week.

During the first fourteen weeks lamb 839 lost thirty pounds but from this time till the end of the experiment it put on weight, regaining a total of thirteen pounds (Fig. 7). After the tenth week this lamb developed an oligocythaemia, but the condition did not become so severe as in the lambs which died.

Lamb 854 showed only very slight effects, losing some five pounds in weight during the experimental period, but showing no pathological changes in its blood picture (Fig. 8).

DISCUSSION

From the results it is clear that not all the experimental lambs were affected in the same way although all received a similar number of infective larvae and similar treatment throughout the experimental period. The differences observed are doubtless referable to differing degrees of inherent resistance in the individual sheep. The three lambs which died all had high worm burdens, 55,700, 23,650 and 51,000 respectively at death, and had steeply rising egg counts throughout the experimental period, indicating that they were very susceptible to infestation and that they did not develop resistance. As a result of the heavy worm burden the symptoms shown by these lambs were severe. The two lambs which were killed at the end of the experiment carried worm burdens of 6,800 and 17,000 respectively and their egg counts, after an initial steep rise, fell rapidly and then remained at a low level. These lambs were resistant to infestation and the symptoms shown by them were considerably less severe than those seen in the lambs which died. The third lamb of this group was, in fact, sacrificed during the course of the experiment along with a control for examination of the lesions present at that particular point in the experiment. The worm burden of this lamb was high (57,000). It is debatable whether the animal was slaughtered a little before its resistance would have enabled it to throw off its worm burden or whether the high worm burden would have resulted in death. The fact that its egg count rose initially and

then fell to a low level indicates that the former explanation is the more likely.

The difference in the reaction of individual sheep to infection is, of course, well known and was remarked on by Fourie, who in 1931, was one of the first workers to study the effects of pure infestations of trichostrongylid worms in sheep. Fourie, working with *Haemonchus contortus,* observed that of 38 worm-free sheep, artificially infected with *H. contortus,* only nine developed a fatal anaemia, six developed an anaemia from which they recovered and twenty-three showed no effects whatsoever. This is a common experience, having been recorded by many other authors and is one of the major difficulties in work of this type.

The anaemia (oligocythaemia) produced by *T. axei* in lambs seems to be somewhat unusual in that regenerative forms are absent. It appears to be entirely caused by a failure in the production of erythrocytes by the erythroblastic tissues, the normal destruction of red cells resulting in a gradual decrease in the total number of erythrocytes in the blood. There is no change in average cell size or cell haemoblobin content as revealed by the values for mean corpuscular volume, and mean corpuscular haemoglobin content respectively. It differs from the anaemia produced by *Haemonchus contortus* in which the red cells increase in size. In the opinion of Fourie (1931) the changes in the latter were identical with those occurring in a sheep bled from the jugular vein until a severe anaemia developed. Andrews (1942) also concludes that the anaemia caused by *H. contortus* is due to haemorrhage. Since *T. axei* has not been shown to be a blood-sucker and the anaemia it produces is different from that produced by *H. contortus* it seems likely that *T. axei* produces its effects either by an interference with the blood-forming cells by means of toxins or by upsetting nutrition. Anaemia is not a constant feature of trichostrongylosis, for Andrews (1939) records fatal cases of it in lambs without the appearance of anaemia. Andrews, however, was working with *T. colubriformis* or with a mixture of *T. vitrinus, T. colubriformis* and very few *T. axei.*

He was therefore dealing chiefly with species whose habitat is the small intestine and this may account for the differences between his results and those of the present investigation. In future work it is intended to investigate the pathogenic effects of *T. axei* on sheep kept on a high plane of nutrition and during the course of this work it may become clear whether the anaemia caused by *T. axei* is chiefly nutritional in origin or not.

CONCLUSIONS

Observations were carried out on the effects of a pure infestation of *T. axei* on six eight-month old lambs maintained on a hay diet; two similar lambs were kept as helminth-free controls.

In three of the lambs the infestation proved fatal. The chief

symptoms shown were severe loss of weight, severe oligocythaemia and, just before death, a tendency to neutrophilia.

The other three lambs were less severely affected and survived until they were killed for post-mortem examination. One of these, killed about the middle of the experiment, showed only slight loss of weight and no change in the blood picture. In the other two lambs, slaughtered at the end of the experiment, changes in the weight and blood picture were insignificant.

ACKNOWLEDGMENTS

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IMMUNOLOGICAL STUDIES ON THE INFECTION OF OVINE ENZOOTIC ABORTION IN YOUNG LAMBS

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INTRODUCTION

Ewe lambs born in infected flocks may be infected with the virus of abortion which causes abortion or infection of the foetal membranes at their first pregnancy (McEwen, Littlejohn and Foggie, 1951). The infection of the lambs may occur pre- or post-natally. In the latter case it is most probably acquired during the first few days or weeks of life as it is at this time that the lambs are exposed to a heavy dissemination of the virus.

It has been shown that ewes infected at one pregnancy are usually free from demonstrable infection at the next and furthermore that during this pregnancy they are resistant to the inoculation of virus (McEwen, Stamp and Littlejohn, 1951). The question was raised whether the lambs from previously infected but immune ewes were themselves resistant to infection because of a passive immunity acquired from their dams.

Part 1 of the present paper deals with the immunological status, as assessed by the presence of complement-fixing antibodies in the sera of lambs born to previously infected but immune ewes, and by the resistance of such lambs to the inoculation of virus.

Part 2 deals with the source of the complement-fixing antibodies present in lambs' sera.

METHODS

The technique used in the complement-fixation test was as follows :—Dilutions of 1/5 of the sera were inactivated by heating at 60°C. for 30 minutes. Serial doubling dilutions of sera from 1/5 onwards were set up. One volume containing 2 M.H.D. of complement and one volume of heated foetal membrane antigen (Stamp, Watt and Cockburn, 1952) were added to each tube and the tubes were retained in the cold store overnight. Haemolytic system was added the following morning and the tubes were incubated at 37°C. for one hour. The reading was then made.

Colostrum samples were treated in the following manner :— Rennet (1 per cent by volume) was added to the sample which was then incubated for 1 to 2 hours at 37°C. The whey was centrifuged to remove traces of clot. A 1/5 dilution was made and treated in the same manner as the sera.

RESULTS

THE IMMUNE STATUS OF LAMBS FROM PREVIOUSLY INFECTED EWES

Three groups of ewes were used in the first season of the experiment. The first (Group A) were ewes from an experimental flock that had aborted or lambed with infected membranes in the spring of 1951 when the animals were just over one year old. At that time the degree of infection of the foetal membrances had varied greatly, the membranes from some animals being extensively diseased whereas others showed but a light infection (McEwen, *et al.*, 1951). The second group (B) were infected ewes of different ages that had aborted in the field at the previous pregnancy and were later purchased for experimental work. The controls (Group C) were gimmers (virgin ewes in their second year) purchased from a farm on which enzootic abortion is not known to occur.

The three groups of ewes were run together with four rams from 23rd August to 2nd October, 1951. In Group A, 22 ewes became pregnant, in Group B, 19 ewes became pregnant, and in Group C, 25 ewes became pregnant. The ewes were brought in from pasture and each group was housed separately on the 17th of December.

As each ewe lambed or was about to lamb she was removed from her group and isolated in a loose box. The foetal membranes were collected and examined macroscopically and microscopically for evidence of infection with ovine abortion virus. If this examination proved negative the ewe and her progeny were retained in the box for two or three days and then turned out to pasture along with the healthy ewes and lambs from all the groups.

None of the ewes aborted and only one had infected membranes. This ewe belonged to Group A and lambed five days before the estimated lambing date. Her foetal membrances were heavily infected with enzootic abortion virus. Her lamb, a male, was small but active. This animal and her lamb were kept isolated.

Serum samples, taken on the 29th October, between 22nd December and 18th January, and from each ewe and her progeny between 7 days and 21 days after lambing (in February), were examined for complement-fixing antibodies. The results of the tests on the ewe sera (Table I) show that by the winter following an abortion or an infected lambing the titres of the ewes' sera were not high. Stamp *et al.* (1952) take a titre of complete fixation at 1/16 or over as indicating infection in ewes in the field. Taking complete fixation at 1/20 as the index of positive reaction, less than half of the ewes in Groups A and B would be classified as positive reactors on any of the three occasions on which they were tested. The ewe which lambed with infected membranes was the only high positive in Group A. In Group B titres of 1/80 or over were recorded on four occasions. The titres of the control ewes (Group C) remained low throughout the experiment.

TABLE I

TITRES OF EWES TO COMPLEMENT/FIXATION TEST

Group	*Date of bleeding*	*Number of samples*	*Numbers of samples giving complete fixation at dilutions of:*									
			<1/5	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
A. previously infected spring 1951	29th Oct. 1951	17	4	8	2	2	1*	0	0	0	0	0
	Dec. 1951—Jan. 1952	22	10	3	4	5*	0	0	0	0	0	0
	Feb. 1952 after lambing	22	4	3	5	6	3	0	0	0	0	1*
B. infected abortion in field spring 1951	29th Oct. 1951	5	1	2	0	0	1	1	0	0	0	0
	Dec. 1951—Jan. 1952	12	2	0	4	2	2	2	0	0	0	0
	Feb. 1952 after lambing	12	6	2	0	2	1	0	1	0	0	0
C. uninfected controls	29th Oct. 1951	11	11	0	0	0	0	0	0	0	0	0
	Dec. 1951—Jan. 1952	23	19	2	2	0	0	0	0	0	0	0
	Feb. 1952 after lambing	23	20	3	0	0	0	0	0	0	0	0

*Titre of ewe which was infected at parturition.

Table 2, giving the distribution of the serum titres of lambs from the ewes in Groups A, B and C within three weeks of birth and at four months of age, shows that with a few exceptions young lambs from previously infected dams had positive titres while the lambs from the control ewes were negative. (Statistically, taking titres of 1/20 and over as positive and titres of less than 1/20 as negative, there is a very highly significant difference of $\chi^2 = 51{\cdot}45$ between the two groups.)

TABLE 2

TITRES OF LAMBS TO COMPLEMENT/FIXATION TEST

Date of bleeding	*Group*	*Number of samples*	*Number of samples giving complete fixation at dilutions of:*						
			<1/5	1/5	1/10	1/20	1/40	1/80	1/160
Feb. 1952 within 3 weeks of birth	A	25	0	0	1	7	7	7	3
	B	17	1	1	2	6	4	3	0
	C	30	26	3	1	0	0	0	0
June 1952	A	24	17	7	0	0	0	0	0
	B	7	7	0	0	0	0	0	0
	C	23	13	6	4	0	0	0	0

In order to determine whether the immunity indicated by these raised titres would protect lambs against infection, all the ewe lambs were inoculated subcutaneously with a dose of 1/1000 suspension of infected yolk sac when less than one month old. This injection had little effect on the titres of the lambs and by June the titres of both ewe and ram lambs had dropped to a low level.

The ewe lambs from both the previously infected ewes and from the non-infected ewes, which for convenience will be called "immune hoggs" and "non-immune hoggs" (a hogg being a sheep between weaning and 15 months old) were run with the ram from the end of November, 1952 until the end of February, 1953. Fertile mating occurred in 19 of the "immune hoggs" but only 6 of the "non-immune hoggs" became pregnant.

Blood samples were taken from these animals at intervals before and during pregnancy and within a month after they had lambed or aborted. With two exceptions, in which the placentas were lost, the foetal membranes from each hogg were examined macroscopically and microscopically for evidence of infection with the enzootic ovine abortion virus. The complement-fixation titres throughout the experiment and the mode of termination of pregnancy of these sheep are shown in Tables 3 and 4.

TABLE 3

COMPLEMENT/FIXATION TITRES OF INFECTED EWE LAMBS FROM INFECTED EWES AND MODE OF TERMINATION OF PREGNANCY

Number of animal	*Feb.*	*June*	*Oct.*	*Jan.*	*March*	*Termination of pregnancy*	*Examination of foetal membrane*	*Titre in June*
2601	1/160	—	1/5	1/5	1/5	Aborted	+	1/640
2607	1/80	—	1/5	1/10	1/10	,,	+	1/20
2606	1/80	—	1/20	1/20	1/80	Lambed	+	1/640
2667	1/20	—	1/10	—	1/20	,,	+	1/640
2605	1/80	—	1/40	1/20	1/5	,,	—	1/320
2620	1/20	1/5	1/20	1/10	1/20	,,	—	1/160
2622			—	1/5		,,	—	1/160
2596	1/80	1/5	1/40	1/20	1/40	,,	—	1/80
2644	1/10	—	1/5	1/10	1/5	,,	—	1/20
2585	1/40	—	—	—	—	,,	—	1/10
2603	1/40	—	—	—	1/5	,,	—	1/10
2610	1/80	—	1/5	1/10	1/5	,,	—	1/10
2591	1/80	—	1/10	1/5	—	,,	Lost	1/10
2583	1/40	—	1/5	1/5	1/5	,,	—	1/5
2599	1/80	1/5	1/20	1/20	1/5	,,	—	1/5
2653	—	—	1/10	1/10	1/20	,,	—	1/5
2632			1/10	1/5	—	,,	—	1/5
2635		—	1/40	1/10	1/5	,,	—	1/5
2616	1/40	—	1/5	1/5	1/10	,,	Lost	—

TABLE 4

COMPLEMENT/FIXATION TITRES OF INFECTED EWE LAMBS FROM UNINFECTED EWES AND MODE OF TERMINATION OF PREGNANCY

Number of animal	*Feb.*	*June*	*Oct.*	*Jan.*	*March*	*Termination of pregnancy*	*Examination of foetal membrane*	*Titre in June*
2645	—	1/5	1/20	1/20	1/40	Aborted	+	1/640
2593	—	1/5			—	,,	—	1/1280
2639	—	1/10	1/10	1/40	1/80	Lambed	+	1/1280
2640	—	—	1/20	1/10	1/20	,,	—	1/160
2646	—	1/10	1/20	1/20	1/40	,,	—	1/20
2641	1/5	—	1/20	1/20	1/80	,,	—	Killed Prolapsed Uterus

Thus, of the 19 "immune hoggs", 2 aborted, 2 lambed with infected membranes, and 15 lambed normally. Of the 6 "non-immune hoggs", 2 aborted, 1 lambed with infected membranes and 3 lambed normally. In one of the "non-immune hoggs", which aborted, the membrane was infected. In the other (No. 2593) the membrane was retained and the virus was not demonstrated in the uterine discharge. The high post-parturient complement-fixation titre of this hogg, however, indicates that an active infection had been present and was probably the cause of abortion.

Although the proportion of abortions and infected lambings in

the "immune hoggs" is smaller than those in the "non-immune hogs" the difference is not significant ($\chi^2 = 1{\cdot}894$, $P = 0{\cdot}2$ to $0{\cdot}1$).

Some points of interest arise from the study of the complement-fixation titres of these animals. As already noted the high initial titres of the lambs from previously infected mothers had dropped to a low level by June, 1952. Thereafter, a number of the animals in both groups showed low positive or doubtful titres until March, 1953. These reactions were presumably the result of the injection of virus and appeared more consistently in the "non-immune hoggs". There is, however, no correlation between those pre-parturient raised titres and the occurrence of abortion or infected lambing, and it would appear that the complement-fixation test is of little value in making a prognosis as to the manner in which pregnancy will terminate.

There is, however, a high degree of correlation between the presence or absence of abortion or infected lambing and the titres of serum samples taken within a month after lambing. Five of the six hoggs which aborted or were infected at lambing had titres of 1/640 or over while none of the 18 hoggs which lambed normally had a titre greater than 1/320.

THE TRANSFERENCE OF COMPLEMENT-FIXING ANTIBODY FROM EWE TO LAMB VIA THE COLOSTRUM

On the basis of the examination of 12 sera from lambs suckling infected foster mothers, Stamp *et al.* (1952) suggest that complement-fixing antibodies are not passed via the colostrum from ewe to lamb. If this conclusion were correct it would be difficult to explain the findings described above (Table 2) where of 42 lambs from previously infected mothers, 37 had titres greater than 1/10 while of 30 lambs from uninfected mothers none had a titre greater than 1/10.

The following experiments were carried out in order to determine whether colostral transference of immunity does, in fact, take place :—On March 20th, 1953, blood samples were taken immediately after birth from 22 lambs in a flock where the disease is endemic. The lambs were ear-tagged at birth and a second sample was taken from the 19 surviving lambs and from their mothers on 4th April, 1953. The results of the complement-fixation test are given in Table 5. All the lambs were negative to the test at birth. Although none of the ewes were positive, 5 of the lamb sera gave complete fixation at 1/20 when 15 days old.

The foetal membrane vaccine reported on by Littlejohn, Foggie and McEwen, 1952, although giving a good field protection, is known to have little effect on the complement-fixation titre of vaccinated sheep. More recent tests (McEwen and Foggie, publication pending) have, however, shown that a high complement-fixation titre can be expected to result from the injection of alum-

precipitated adjuvant vaccines. This reaction was used in the following experiment.

A group of eleven ewes in a breeding experiment had been inoculated with ordinary foetal membrane vaccine to protect them against accidental infection with enzootic ovine abortion virus. These ewes were bled on 17/2/53 and inoculated on the same day with 2 c.c. adjuvant vaccine. On 19/3/53 further blood samples were taken and 1 c.c. adjuvant vaccine inoculated into each ewe. A final

Table 5

Complement/Fixation Titres of Ewes, and Lambs before and after Sucking, in Infected Flock

	Number of samples	*Number of samples giving complete fixation at dilutions of:*			
		<1/5	1/5	1/10	1/20
Birth	22	22	0	0	0
15 days	19	6	8	0	5
Ewes	11	6	5	0	0

blood sample was taken in May between one and two weeks after lambing. The results of the complement-fixation test on sera from these samples is given in Table 6. Thus, at lambing these ewes had

Table 6

Complement/Fixation Titres of Immunized Ewes

Date	*Number of samples*	*Number of samples giving complete fixation at dilutions of:*									
		<1/5	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
17/2/53 ..	11	7	3	0	0	0	1	0	0	0	0
19/3/53 ..	11	1	0	2	0	2	1	4	1	0	0
May, after lambing ..	11	0	0	0	0	2	5	2	0	1	1

a high titre of complement-fixing antibodies. Blood samples were obtained from 13 of their lambs before they had sucked. A further 9 lambs were first bled after they had sucked. Blood samples were taken from these lambs periodically for three months. The results

of the complement-fixation test on the lambs' sera are given in Table 7.

TABLE 7

COMPLEMENT/FIXATION TITRES OF LAMBS FROM IMMUNIZED MOTHERS

Age	*Number of samples*	*Number of samples giving complete fixation at dilutions of:*										
		<1/5	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Birth ..	13	13	0	0	0	0	0	0	0	0	0	0
1 week	18	0	0	0	1	0	3	0	7	4	1	2
2 weeks	17	0	0	1	0	4	3	4	2	1	2	0
3 weeks	17	0	0	1	3	5	6	0	0	2	0	0
4 weeks	18	0	1	1	3	5	5	1	0	2	0	0
5-7 weeks	18	0	1	4	5	3	3	0	0	2	0	0
8-10 weeks	13	1	6	1	1	4	0	0	0	0	0	0
11-13 weeks	19	6	8	3	2	0	0	0	0	0	0	0

These results show that lambs from ewes immunized with adjuvant vaccine are born with a negative complement-fixation titre but rapidly acquire a high titre after they have sucked. A pair of twin lambs bled 16 hours after birth had titres of 1/1280 and 1/640 respectively. The high titre present within 1 week of birth declines over a period of 3 months to a low level. The amount of antibody present at any given period appears to depend on the height of the initial titre.

Samples of colostrum from two of the ewes were tested for complement-fixing antibodies. Both these samples were anti-complementary to a titre of 1/40 but in the presence of antigen they fixed complement to titres of 1/5120 and 1/640 respectively, showing that the colostrum of immunized ewes is rich in complement-fixing antibody.

The group of ewe hoggs which had been infected shortly after birth and mated in their first year of life (Table 3) gave a picture clinically closely resembling a naturally infected flock. As the lambing in this group spread over 2 months, it did not prove possible to bleed each lamb at birth, but when opportunity arose samples were taken from newly born lambs before they had sucked. Table 8 shows the complement-fixation titres of the 16 lambs from ewe hoggs which lambed normally with uninfected membranes. As might be expected from the post-parturient titres of the dams (Tables 3 and 4) there was a much lower level of immunity in this group of lambs and the titres dropped to a low level in 7 to

TABLE 8

COMPLEMENT/FIXATION TITRES OF LAMBS FROM EWE HOGGS WHICH LAMBED NORMALLY

Age	Number of samples	Number of samples giving complete fixation at dilutions of:						
		<1/5	1/5	1/10	1/20	1/40	1/80	1/160
Birth	5	5	0	0	0	0	0	0
1 week	12	2	4	1	1	0	3	1
2 weeks	10	2	4	1	3	0	0	0
3-4 weeks ..	9	0	4	1	3	1	0	0
5-6 weeks ..	10	5	3	1	1	0	0	0
7-8 weeks ..	8	7	1	0	0	0	0	0
9 weeks	6	6	0	0	0	0	0	0

8 weeks. In more than half of the lambs sampled there appeared to be very little increase of titre after sucking. Colostrum samples from two of the ewes in this group were tested and found to have titres of 1/5 and less than 1/5 respectively. The titres of their lambs remained low. A colostrum sample from a third ewe had a titre of 1/320. The titre of her lamb after sucking was 1/80.

Three live lambs were born from dams with heavily infected membranes. Ewe hogg 2677 had a titre of 1/20 in March. She gave birth to a strong lamb in heavily infected membranes. The dam's titre at parturition was 1/80 and rose to 1/640 by 12th June. A sample of colostrum had a titre of 1/80. A blood sample from the lamb before sucking gave a negative titre. The titre rose to 1/40 after sucking. Ewe hogg 2606 had a pre-parturient titre of 1/80, and a post-parturient titre of 1/640. On May 13th she gave birth to a small weak lamb with heavily infected membranes. The lamb was seen within one hour of birth when it was in a collapsed condition and was unable to stand. It is extremely unlikely that this lamb had sucked the ewe before a blood sample had been taken as it remained weak and had to be held to the udder to feed for the following 7 days after which it was destroyed. The initial titre of this lamb in what is believed to have been a pre-sucking sample was 1/20. The following day after the lamb had sucked the titre rose to 1/1280 and was 1/640 when the lamb was slaughtered. A colostrum sample from the ewe gave a titre of 1/2560. If the evidence that this lamb had not sucked prior to bleeding is accepted, the titre of 1/20 in the initial sample raises two possibilities. Either the lamb had responded to the infection of its membranes by the production of an active immunity or antibodies from the ewe's serum

had passed through the damaged membranes to the foetal circulation. This prenatal immunity, if it does occur however, remains insignificant compared with the transference of antibody resulting from the ingestion of immune colostrum. A third ewe hogg (No. 2639) lambed with infected membranes and a post-parturient titre of 1/1280. Her lamb was bled 2 hours after birth. Unfortunately the serum was anticomplementary to a titre of 1/10 and did not fix complement at higher dilutions. The lamb's titre after sucking was 1/640.

Ewe hogg 2607 aborted a dead, nearly full term foetus with infected membranes. Serum obtained from the heart blood showed no complement-fixing antibody at dilutions of 1/5 or higher.

DISCUSSION

The value of the maternal immunity received from the colostrum in protecting lambs from an experimental neo-natal infection with a large dose of virus was not demonstrated in the experiment in part 1. Although there was a greater proportion of infected pregnancies in the group of ewe hoggs from uninfected mothers the total number of animals in this group was small and the result is not significant.

In the second group of experiments the source of the raised titres in young lambs was examined and it appears from the evidence presented that this resulted from complement-fixing antibody absorbed from the colostrum. There is, however, the possibility that lambs from infected ewes may have some degree of immunity before they have ingested colostrum. The source of this immunity is at present hypothetical.

As is shown in Table I the titres of ewes previously infected may have dropped to an indeterminate level and the presence or absence of a previous infection may be best deduced from the titres of their lambs after they have sucked (Table II). From the evidence here presented it would appear that the optimum time to take serum samples is within a month or two of infected lambing or abortion. As antibody may not be demonstrable in the serum until shortly before or even after parturition the test gives little information as to the probable termination of a current pregnancy.

SUMMARY

Lambs from ewes which had been infected with ewe abortion virus at the previous pregnancy, and from vaccinated ewes, were found to have positive titres to the complement-fixation test when less than three weeks old.

This positive titre resulted from the absorption of complement-fixing antibodies from the colostrum.

Lambs from uninfected mothers had negative titres.

There is a possibility that some lambs born with infected membranes may have some immunity prior to the ingestion of colostrum.

Experimental infection of ewe lambs from immune mothers failed to demonstrate that they were protected against abortion at their first pregnancy.

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CARRIERS OF SWINE ERYSIPELAS

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INTRODUCTION

Erysipelothrix rhusiopathiae appears to have a wide distribution in nature and it has been recovered from a variety of sources such as putrefying material, especially meat, from preserves, shellfish, fish, sheep, poultry and, in areas where swine erysipelas exists, from the soil (Hutyra, Marek and Manninger, 1946; Kondo and Sugimura, 1935; Kelser and Schoening, 1948). Apart from the acute and subacute forms of swine erysipelas, the organism is a well-known cause of chronic infection in pigs and it has been isolated from the affected joints in a high proportion of cases of arthritic conditions in these animals (Connell, Moynihan and Frank, 1952). It seems that it can also exist more or less as a commensal within some pigs without causing any obvious ill-effects and several Continental investigators have demonstrated its presence in the tonsils and intestines of apparently normal animals (Pitt, 1908; Bramm, 1937).

It was thought that it might be of interest to ascertain to what extent such "carrier" pigs occurred in this country but instead of tonsils and intestines, to use bone marrow as material for examination. Arrangements were made, therefore, with a London slaughterhouse to send a long bone from each of several hundred pigs, selected at random over a period, from the animals sent there for slaughter. A total of 348 femurs from individual pigs were received during a period of about ten months and these were examined for the presence of erysipelothrix organisms by cultural and biological methods. It is understood that all the animals from which these bones were removed were about eight months of age. One half of the bones came from fresh carcases and the remaining half from salted ones. None showed any arthritic lesions on the articular surfaces.

TECHNIQUE

Attention was confined to the red marrow of these bones. Each bone was flamed to reduce extraneous infection and then opened with a sterile saw. As much red marrow as possible was removed with a clean spatula and transferred to a mortar.

Method A

The first 204 bones were examined on the assumption, later proved wrong, that if erysipelothrix organisms were present in them,

they would be numerous enough and of sufficient pathogenicity to kill mice inoculated with a saline suspension of the marrow. The red marrow from each bone was ground up with 10 ml.of normal saline and a small quantity of sand and then filtered through muslin to remove coarse particles. About 0·25 ml. of this filtrate was plated on selective medium containing 1/1,000 sodium azide and 1/100,000 crystal violet (Packer, 1943). At the same time, two to four white mice were each inoculated subcutaneously with 0·5 ml. of the filtrate from each bone marrow suspension. The plates were incubated for 48 hours at 37·5°C. and then examined for colonies containing slender, Gram-positive bacilli. The mice were kept for fourteen days and any dying during that time were examined by smear preparation and culture of the spleen for the presence of *E. rhusiopathiae*. In cases where the mice died and proved positive on examination, a pigeon was inoculated with 1 ml. of the same filtrate used to inoculate the mice. This was intended as additional evidence that the organisms were present in the filtrate and had not been reactivated from some latent infection in the mice themselves.

TABLE I
METHOD A

Number of Bones Examined	*Number Infected with Erysipelothrix Type Organisms*							
	Positive on Culture		*Confirmed by Biological Test*					
			In Mice and Pigeons		*In Mice Only*		*Total Confirmed Biologically*	
	No.	%	*No.*	%	*No.*	%	*No.*	%
102 (fresh)	22	21·5	2	2	4	3·9	6	5·9
102 (salted)	12	11·8	1	1	4	3·9	5	4·9
204	34	16·7	3	1·5	8	3·9	11	5·4

Results with method A. These are set out in Table I and it will be seen that although it appeared by cultural examination that 34, or 16·6 per cent, were positive, in only eleven cases did the inoculum prove fatal to mice and in only three of these did it also kill the pigeon. In fact, there was every indication that the mice were more susceptible to infection than the pigeons. Breed (1942) has commented on instances in which organisms isolated from sick and dying pigs, though identical in every other way with *E. rhusiopathiae*, would not kill pigeons even if inoculated into them in large numbers.

Method B.

It was noted in Method A that in many cases where the marrow suspension proved positive on culture but not on biological tests,

the organisms were not only few in number in the suspension but appeared to have a rough colonial formation on culture and were of reduced pathogenicity for both mice and pigeons. It was decided, therefore, to alter the technique slightly in order to examine the remaining 144 bones. The same procedure was followed as in Method A except that instead of suspending the marrow in saline, a broth containing sodium azide and crystal violet was used and the suspension incubated for 48 hours before testing it. It was then plated out as in Method A and also inoculated into groups of two to four white mice, each being given 0·25 ml. subcutaneously.

TABLE 2

METHOD B

Number of Bones Examined	*Number Infected with Erysipelothrix Type Organisms*							
	Positive on Culture		*Confirmed by Biological Test*					
			In Mice and Pigeons		*In Mice Only*		*Total Confirmed Biologically*	
	No.	%	*No.*	%	*No.*	%	*No.*	%
72 (fresh)	50	69·4	25	34·7	9	12·5	34	47·2
72 (salted)	26	36·1	6	8·3	12	16·7	18	25·0
144	76	52·8	31	21·5	21	14·6	52	36·1

Results with method B. This seemed to be a more sensitive method; apart from the erysipelothrix, the only other organisms which grew freely were streptococci which did not appear to affect the mice unduly, and there was less discrepancy between the results obtained by cultural test and those by biological test. (Table 2). A higher proportion of fresh bones were positive than those from salted carcases. It may be of interest to note that all the mice which died and proved positive on post-mortem examination developed a severe conjunctivitis before death, a symptom which is often associated with the disease in these animals. There was no evidence that any latent infection with erysipelas existed in the stock from which these mice were drawn.

DISCUSSION

These findings agree generally with those of other investigators and it appears that a high proportion of apparently healthy pigs may harbour organisms indistinguishable from *E. rhusiopathiae* without showing any noticeable ill-effects, at least in early life. In view of the apparent wide occurrence of the organism in nature, this may not be altogether surprising and a great number of pigs must

at some time be hazarded to infection. It is possible, of course, that in some of the pigs the infection was derived from vaccine administered earlier. It is also just possible that the infection did not exist in the bone marrow during life but spread to this site from the alimentary tract after death.

It is difficult to assess the significance of such a high number of carriers in relation to the general health of the pig population but no doubt many of them would remain throughout their lives unaffected to any extent by the presence of these organisms, either in the alimentary tract or elsewhere; whether they could depress the general level of health so slightly as to remain unobserved or not is problematical. According to Fulton (1933), infection may exist in a herd for a long time without any clinical manifestations of the disease except for a retarding effect on the growth of young pigs. If this is correct, it is interesting to speculate to what extent the beneficial effect on the growth rate of pigs of feeding antibiotics may be attributable to the sensitivity of erysipelothrix to these substances.

The inoculation of avirulent or attenuated vaccines, not requiring the simultaneous use of antiserum, into such carrier animals might present some difficulties for, though the vaccines themselves may be incapable of setting up the disease, it is possible they might reactivate the latent infection and give rise to the clinical form of the disease. In the well established serum-culture method of vaccination, the protective effect of the serum would probably prevent such a reactivation.

CONCLUSIONS

The red marrow of each of 348 long bones, selected at random from individual pigs killed at a slaughter-house, was examined by culture and by biological tests in mice and pigeons for the presence of *E. rhusiopathiae*.

By one method, later found to be insufficiently sensitive, 34 of 204 bones (16·7 per cent) were positive on culture but only eleven of these (5.4 per cent) were confirmed by biological tests.

By a second and more sensitive method, 76 of 144 bones (52.8 per cent) were found positive on culture and of these, 52 (36·1 per cent) were confirmed by biological tests.

Many of the strains of erysipelothrix recovered were in the R phase and were of reduced pathogenicity for mice and pigeons. Mice appeared to be more susceptible to infection than pigeons.

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EXPERIMENTAL MAGNESIUM DEFICIENCY IN CALVES

I. Clinical and pathological observations

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INTRODUCTION

In 1923 McCandlish suggested that the nutritive failure of calves given whole milk for very long periods was related to their metabolism of magnesium. It was subsequently demonstrated by Duncan, Huffman and Robinson (1935) that the magnesium content of the blood serum of calves fell when they were given rations of whole milk supplemented with iron, copper and manganese salts and cod-liver oil. It was estimated that 30 to 40 mg./kg. body weight was necessary to maintain normal plasma concentrations of magnesium when a supplement was given as magnesium salts, whereas an intake of 12 to 15 mg./kg. body weight was sufficient when the source of the dietary magnesium was natural feedingstuffs (Huffman, Conley, Lightfoot and Duncan, 1941). This comparison of the effectiveness of natural feeds and magnesium salts suggests that magnesium deficiency was not the sole factor concerned in the development of the syndrome. That supplements of magnesium, together with adequate amounts of iron, copper and vitamin D prevent many, but not all, of the signs of nutritive failure when calves are given whole milk for periods longer than 3 months has been demonstrated in several experiments (Herman, 1936; Knoop, Krauss and Washburn, 1935; Wise, Gullickson and Petersen, 1939; Wise, Petersen and Gullickson, 1939).

In the studies of the gross and microscopic pathology of the "milk syndrome", the presence of calcified plaques on the endocardium and the wall of the aorta, the general calcification of soft tissues, and the presence of nephritis were particularly striking. A marked hyaline degeneration of the skeletal musculature with concomitant calcification was also observed (Moore, Sholl and Hallman, 1936; Moore, Hallman and Sholl, 1938) and from the published microphotographs the lesions appear to be almost identical with those observed in experimental vitamin E deficiency in the naturally occurring muscular dystrophy, weisses Fleisch, and in cod-liver oil

poisoning (MacDonald, Blaxter, Watts and Wood, 1952; Blaxter, Wood and MacDonald, 1953). Sjollema (1935) has also stressed the degeneration of the musculature and the presence of intramuscular oedema which occurs in a comparable "milk syndrome" in Holland. Moreover, many of the clinical signs exhibited in the disease are indistinguishable from those known to occur in muscular dystrophy (cf. McCandlish, 1923; Cannon, 1931; Wise, *et al.*, 1939b). Since in the experiments of Duncan, *et al.* (1935), which provided the material for Moore, *et al.* (1938), cod-liver oil was included in the ration, it is possible that dystrophic degeneration due to cod-liver oil poisoning complicates the interpretation of the pathological findings.

In the experiments of Huffman, *et al.* (1941) the requirement of a milk-fed calf for dietary magnesium is placed at 30 to 40 mg./kg. body weight per day, which, assuming an intake of milk equivalent to 12 per cent of body weight, is equivalent to a concentration of magnesium in the dry matter of the diet of 2,000 p.p.m. In rats (Tufts and Greenberg, 1938; Kunkel and Pearson, 1948a) and rabbits (Kunkel and Pearson, 1948b) the dietary requirement necessary to prevent clinical signs lies between 60 and 200 p.p.m., depending on the criteria used to assess adequacy. The apparently much higher requirement of the calf cannot be accounted for by differences in methods of assessment.

In the present series of experiments, an uncomplicated magnesium deficiency has been produced in calves given an artificial diet deficient in magnesium, but including all other known essential nutrients, and the pathology of the disease is reported.

EXPERIMENTAL PROCEDURE

Rations. An artificial magnesium-free basal diet, simulating milk, prepared according to the method of Clark (1927) was used in all the experiments. Its composition is given in Table 1. All mineral salts were analytical reagent grade chemicals, with the exception of the calcium hydroxide which was graded "puriss B.P.". The diet was prepared in batches of 8 gallons (36 l.) at intervals of 4 to 7 days, and preserved by pasteurization and the addition of 1 g. penicillin per 36 l. It was stored at 2°C. The magnesium content of this basal diet determined by chemical analysis was 0·35 to 0·50 mg./100 ml. These analyses were checked spectrographically. Diets with higher magnesium contents of 0·5, 1·6, 4·1, 5·8, 12·5, 19·0 and 24·0 mg/100 ml. were prepared by the addition to the basal diet of a slightly acid solution of magnesium oxide (analytical reagent grade) in concentrated HCl.

Animals. Seven Ayrshire bull calves weighing initially 30 to 35 kg. were used. They were housed in metal lined metabolism cages and were given cow's whole milk for one to two weeks before

Table 1.

Ingredients Used in the Preparation of the Basal Magnesium-low Diet

Ingredient		*Concentration/l*
Light white casein	g.	40
Glucose	g.	30
Margarine	g.	40
Arachis oil	g.	4
Lecithin (egg)	g.	0·5
Calcium hydroxide	g.	1·2
Calcium chloride (anhydrous)	g.	1·56
Calcium carbonate	mg.	140
Sodium hydroxide	mg.	928
Potassium hydroxide	mg.	959
Citric acid	g.	2·19
Sulphuric acid 0·09 N	ml.	30·5
Solution of trace elements*	ml.	5
Potassium dihydrogen phosphate	mg.	873
Vitamin A	i.u.	8000
Vitamin D	i.u.	800
		Daily quantity/calf
α-tocopheryl acetate	mg.	50
Aneurin	mg.	4
Riboflavin	mg.	4
Nicotinic acid	mg.	30
Ca pantothenate	mg.	10
Pyridoxine	mg.	4
Inositol	mg.	200
p-amino benzoic acid	mg.	24
Alcoholic liver extract†	ml.	4
Biotin‡	μg.	4

*One litre of this solution contained Fe citrate 400 mg., $MnSO_4 \cdot 4H_2O$, 20 mg.; $CuSO_4 \cdot 5H_2O$, 100 mg.; $ZnSO_4 7H_2O$, 200 mg.; $CoCl_2 \cdot 6H_2O$, 100 mg.; KI, 80 mg.; NaF, 80 mg.

†A 50% ethyl alcohol extract of fresh liver.

‡Given to calf no. 208 only; subsequently found (Kon & Porter, 1952) not to be required by the calf.

transference to the experimental diets. They had no access to bedding and precautions were taken to prevent their licking the cemented walls of the room through the bars of their cages. The scheme of treatment is given in Table 2.

Methods. The animals were observed closely every day and abnormalities recorded. They were weighed and samples of blood were taken every four days. At death or slaughter, careful post-mortem examinations were made, and samples of certain tissues taken for chemical analysis and histological examination. Metabolic balances were made throughout and will be reported in a later paper (Blaxter and Rook, 1954). The Ca, Mg and inorganic P

Table 2.

Scheme of Experimental Treatment

Calf no.	*Date experiment commenced*	*Magnesium content of diets given*		*Fate*
		Preliminary period	*Experimental period*	
	(1952)			
208	July	12·5 mg./100 ml. for 8 days	0·5 mg./100 ml. throughout	Died in convulsions
213	September	12·5 mg./100 ml. for 12 days	1·6 mg./100 ml. throughout	Died in convulsions
214	September	12·5 mg./100 ml. for 12 days	12·5 mg./100 ml. throughout	Survived and slaughtered
215	September	12·5 mg./100 ml. for 12 days	0·5 mg./100 ml. throughout	Killed in convulsions
216	September	12·5 mg./100 ml. for 12 days	5· 8 mg./100 ml. for 1st 52 days subsequently 4·1 mg./100 ml.	Survived and slaughtered
	(1953)			
219	January	19·0 mg./100 ml. for 16 days	19·0 mg./100 ml. for 1st 40 days, subsequently 24·0 mg./100 ml.	Survived and slaughtered
220	January	19·0 mg./100 ml. for 16 days	0·5 mg./100 ml. throughout	Slaughtered after 109 days of treatment (see text)

contents of blood were determined by the methods described by Godden (1935). Bones and soft tissues were dried, ashed at low temperature and the Ca and Mg were determined by modifications of the method of McCrudden (See Hawk, Oser and Summerson, 1947). The manganese content of the materials was low and did not interfere in the determination of Mg.

RESULTS

Clinical Signs

Calves 214 and 219, which received diets containing 12·5 and 19·0 mg. Mg/100 ml. diet respectively and calf 216, which received 5·8 and later 4·1 mg./100 ml. diet, appeared perfectly normal throughout. The remaining four calves, three of which received the basal diet (0·5 mg./100 ml.) and the other, calf 213, a diet containing 1·6 mg./100 ml. all showed clinical signs of magnesium deficiency. With these calves nothing abnormal was noted until the blood serum magnesium had fallen to below 0·7 mg./100 ml. Careful watch was made for the signs of the hyperaemia and vasodilation observed in experiments with rats (McCollum and Orent, 1931; Watchhorn and McCance, 1937; Schrader, Prickett and Salmon, 1937) during the early stages of the production of the deficiency. They were not observed, nor were the loss of hair and skin changes noted by the above workers.

The first unequivocal signs to be seen were changes in the carriage of the head and ears. In all calves opisthotonus was observed, and was most severe in calf 208. A photograph is shown in Plate I. The opisthotonus in calf 213 was exhibited spasmodically fairly early in the experiment and tended to increase in severity with time. It was found necessary to watch the calves for long periods in order to see opisthotonus in its early stages, since the mere presence of an observer tended to distract the calves' attention at this time. The head retraction was at times more to one side than another, but in general it was directly upwards. It was comparable to the opisthotonus of B_1 deficiency, but an increase in the B_1 content of the diet did not result in a decrease in its incidence. Gait was ataxic in calves habitually showing severe opisthotonus, but circling was not observed. Changes in the carriage and movement of the ears were particularly noticeable. They varied slightly from calf to calf. In three of the calves the ears were pressed backwards and slightly downwards and were moved a great deal, which was not in response to noise or movement in the immediate vicinity. With calf 215, the ears were habitually carried in a fallen position, giving the animal the appearance of a Brahmin beast. Plate 2 shows this calf.

At this stage tetany was not present. All the calves were, however, hypersensitive. All superficial reflexes were grossly exaggerated. Thus, scratch reflexes could be elicited by touching the hair of the withers, and resulted in prolonged twitching of the skin to below

the knee and back to the last ribs. Sometimes it would evoke twitching of the skin on the hind limb. Similarly, tapping the facial nerve produced a prolonged flutter of the lower eyelid, and later this could be evoked merely by touching the poll.

At this time the calves developed a behaviour complex best described as apprehensive. When any person, including their attendants, entered the calf-house, they would come to their feet immediately, whereas controls continued to lie quietly; sudden noises resulted in considerable agitation, and at night switching off the light was sufficient to produce a start. The eyes at this time commonly showed a great deal of white, particularly following sudden disturbance, since the eyelids were retracted. Attempts to induce tetany at this stage by excessive and unfamiliar noise were singularly unsuccessful. An immediate agitation was followed by relaxation.

Later, the calves developed fine tremors of the musculature. Skin twitching occurred without any elicitation whatever and tremors could be palpated in the deeper musculature. Kicking at the abdomen was noted, but the pattern of urination and defaecation was unchanged. On exercise the gait was spastic. Even the removal of the calf from its cage was associated with frothing at the mouth and frothing after exercise was particularly marked, long strands of saliva hanging from the mouth, together with copious bubbles. An increase in pulse rate and a slight rise in body temperature occurred at these times.

Finally, the agitation, fine tremors and mild tetany gave way to convulsions. It is not known what factor was responsible for their precipitation, since the daily routine was unaltered. These were observed in two of the calves (208 and 215), but in calf 213 they were presumed to have occurred during the night since it was found dead with evidence of severe struggling. Early stages of the convulsive attacks which lasted for two hours were observed in calf 208. Calf 215 was found in mild convulsions, having been observed as non-convulsive 2 hours previously. In the convulsive stage, gross tetany of the deep muscles was a salient feature. Calf 208 recovered from one such attack, but succumbed to another the following day. Stamping of the feet and violent head retraction heralded the convulsions. The animals fell on their sides with their necks stretched back, the jaws clenched, all respiratory movements abolished and the limbs in violent tonic-clonic convulsions. Placing the calf in the normal recumbent position resulted in a partial relaxation with very heavy respiration, to be followed by a severe repetition, with much grunting. Faecal and urinary incontinence occurred. So violent were the contractions of the jaws that the tongue was severely bruised in both calves 208 and 215. At this stage the pulse rate rose to between 200 and 250/minute. Subsequently the severity of the convulsions diminished, there being marked cyanosis of the mouth and nose, and an uncountable pulse. Death occurred in final convulsions. Plate 3 illustrates the above changes.

During this time the eyes showed a series of changes. Exopthalmos and an increase in the palpebral fissure, gave the animal a very wild appearance, the eye bulging from its socket. Then, at the height of a convulsion, the nictitating membrane, already plainly visible, passed across the eyeball and suddenly the whole eyeball retracted into its socket, giving the calf a dehydrated appearance. This sequence of exopthalmos and endopthalmos occurred throughout the convulsive phase, one timed cycle being 4 minutes.

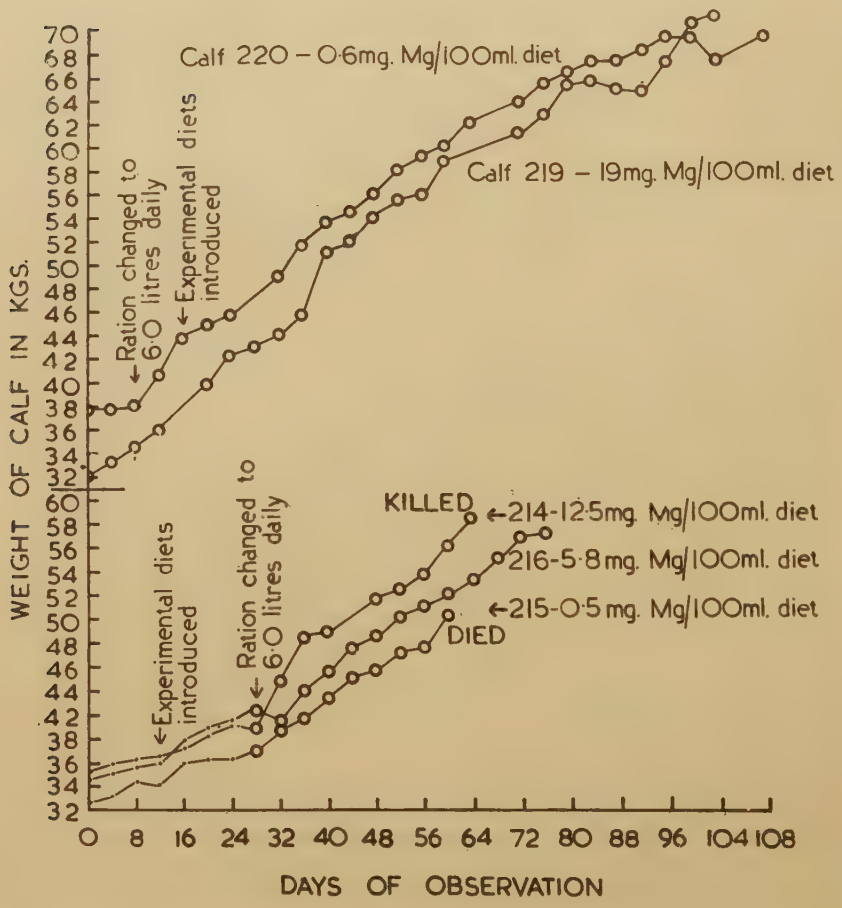
Calves 213, 208 and 215 died or were killed during the first or second convulsive attack. Calf 220 was given 250 mg. Mg as the sulphate intravenously at this time when the blood serum magnesium was 0·51 mg./100 ml. Tetany was not abolished within 2 hours, though the blood level of magnesium was grossly elevated. The blood level had fallen to 1·45 mg./100 ml. within twelve hours. Clinical signs had, however, regressed at this time. Comparative freedom from clinical signs lasted for 3 to 4 days, the blood level having fallen to 0·65 mg./100 ml. within 24 hours of injection, being 0·61 mg. at 48 hours and 0·55 mg. at the end of the 4th day. Subsequently this calf slowly developed what may be termed chronic tetany. Head retraction was shown at almost all times, tetany of the flanks, nostrils, eyelids and shoulders, that is all the superficial musculature, was invariably exhibited, and there was marked ataxia. At this stage marked peripheral neuritis became a prominent sign. The movement of the front limbs in walking was grossly exaggerated, the carpus being bent so that the hoof almost touched the thorax. This is shown in Plate 4. The hind limbs were moved rigidly and here tendon reflexes appeared diminished. Later, relaxation of the pastern joint of the front limbs occurred, resulting in spreading of the claws, the angular displacement being approximately 40°. During walking the heels touched the ground. The sense of balance was clearly affected. The animal would not right itself if laid on its side. Poor balance resulted in much stumbling during walking and bruising of the knees. Sudden frights resulted in collapse with the pulse elevated to 150 to 200/minute, a visible venous pulse, and in fact a visible movement of the thoracic wall over the heart. Paddling of the front limbs, frothing at the mouth, dyspnoea and head retraction, together with diarrhoea and convulsive movements of the abdominal wall, invariably followed. At no time was any sound emitted by the calf during this chronic tetany stage, though prior to this it had been quite vociferous.

This calf was killed when the locomotor signs were advanced. It was taken to the laboratory and the stimulus of the strange environment resulted in violent tetany of all superficial muscles, and the deep muscles of the hind limb. Anaesthesia with pentothal sodium abolished tetany, none being exhibited voluntarily, and none being elicited by touch stimuli.

Bodyweight

Figure 1 shows the body weights of the calves during the course of the experiments. It will be noted that there was very little difference between the calves in their rate of gain in body weight, though there was a slight tendency for those given rations containing more than 10 mg./100 ml. to gain the most. When acute deficiency signs developed, however, there was a distinct reduction in the rate of gain in two of the calves given the ration containing 0·5 mg./100 ml. It may be concluded, therefore, that growth is little affected by subsistence on magnesium deficient rations, at least until severe clinical signs develop.

Fig. 1.

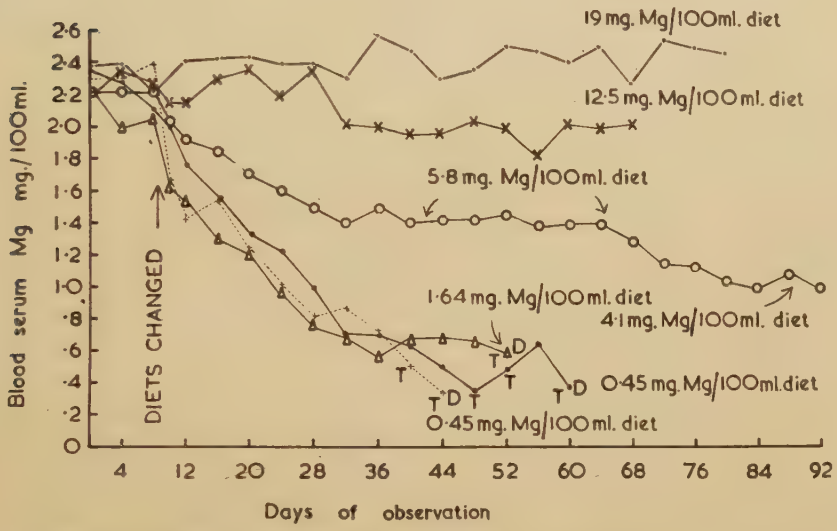


The body weights of calves given diets containing different amounts of magnesium.

Blood Serum Magnesium

In Figure 2 are given the blood serum magnesium concentrations of five of the calves during the experiment. The blood serum magnesium concentrations of the two remaining calves, both given 0·5 mg./100 ml. followed the same course as those for calf 215, included in Figure 2.

Fig. 2.



The concentration of magnesium in the blood serum of calves when given diets containing different amounts of magnesium.

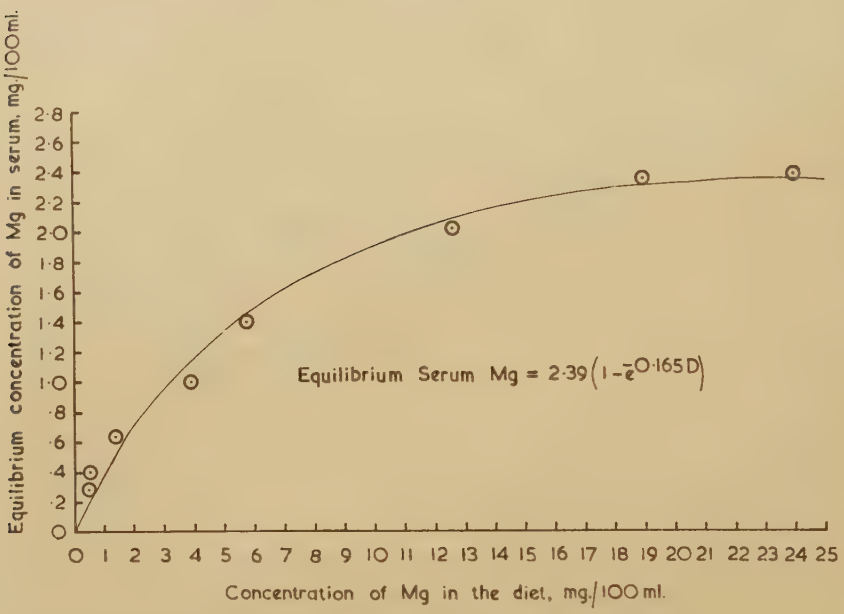
The transference to rations low in magnesium content resulted in a slow exponential fall in blood serum magnesium concentration, an approximate equilibrium being attained after 35 to 50 days, when calves given below 2·0 mg./100 ml. of diet succumbed. With calf 216, reduction of the dietary concentration from 5·8 mg./100 ml. to 4·1 mg./100 ml. led to a further depression of the serum concentration. At no time was a precipitous or sudden fall in concentration observed.

Signs of tetany were not observed until the blood serum magnesium content had fallen to below 0·7 mg./100 ml. With calf 215, an increase in the blood serum magnesium concentration on the 56th day led to a considerable diminution—virtually complete abolition—of clinical signs. The reasons for this rise in the magnesium concentration in the blood is not known. It may have arisen from the breakdown of muscle substance during tetany. In this regard it should be pointed out that the diagnosis of hypomagnesaemia in cows is based on obtaining a value for serum magnesium below 1·7 mg./100 ml. (Allcroft, 1947a) and biochemical

and clinical diagnosis of lactation tetany has in fact been made when the blood serum magnesium has been between 1·6 and 1·7 mg./100 ml. (Allcroft, 1947b). If the field condition observed in cows is comparable to our experimental findings in calves, then it is possible that the relatively high values obtained by Allcroft may reflect the release of magnesium by muscle catabolism during tetany.

In Figure 3, the equilibrium levels of serum magnesium have been plotted against the dietary concentration. Equilibrium levels

Fig. 3.



The relation between the concentration of magnesium in the blood serum of calves 30–50 days after subsistence on diets containing different amounts of magnesium and the dietary concentration of magnesium.

were those obtained 35 to 50 days following a change of diet. The exponential curve describing these data was fitted by least squares and was

$$(Mg)_E = 2{\cdot}39\ (1 - e^{-0{\cdot}165\ D})$$

where (Mg)E represents the magnesium concentration at equilibrium and D the dietary concentration of Mg in mg./100 ml. The equation indicates that in calves given rations simulating milk the maximal concentration of Mg in the serum will not exceed $2{\cdot}4 \pm 0{\cdot}1$ mg./100 ml., however high the dietary concentration. Cow's whole milk contains 7 to 10 mg./100 ml., suggesting that it would not maintain

a concentration of magnesium hitherto regarded as normal (Allcroft, 1947a). From the equation it may be calculated that the serum concentrations would in fact be between limits of $1{\cdot}6 \pm 0{\cdot}15$ and $1{\cdot}9 \pm 0{\cdot}15$ mg./100 ml., that is the lower limit ($P = 0{\cdot}05$) would be 1·3 mg./100 ml. That this is a correct conclusion may be inferred from recent studies (Blaxter and Sharman, 1953) of the blood serum magnesium concentration of calves suckling their dams and receiving virtually no supplementary food. In 24 calves examined at 2-weekly intervals, the mean serum concentration fell from a value of 2·45 mg./100 ml. within a few hours of birth to a mean of 1·74 when the calves were approximately 42 days old. The lowest value obtained was 1·2 mg./100 ml. These calves showed no signs of tetany. It follows therefore that the blood serum magnesium concentration in calves is determined to a large extent by dietary intake and that deviations from the usually accepted normal of 2·00 to 2·50 mg./100 ml. can probably be ascribed to the low magnesium content of the diet.

If it is assumed that the attainment of 90 per cent of the asymptomatic level of blood magnesium (i.e. 2·15 mg./100 ml.) is an indication of a normal saturation of the body with magnesium, then the magnesium requirement of the calf may be calculated to be approximately 13·5 mg./100 ml. milk. Assuming milk to be consumed in amounts equivalent to 12 per cent of body weight this would amount to 16·2 mg./kg. body weight, considerably below the estimates of 40 mg./kg. body weight obtained by Huffman, *et al.* (1941), but, expressed as a concentration of the dietary dry matter (1,080 p.p.m.), considerably above estimates obtained with laboratory species, as previously discussed.

Blood Serum Calcium and Whole Blood Inorganic Phosphorus

The results for blood serum calcium are not given in detail. There was, if anything, a slight increase in the concentration in animals given the diets containing the least magnesium. In calf 208, the terminal tetanic convulsions coincided with a fall in the serum calcium from 11·1 to 8·4 mg./100 ml. No such change was observed with calves 213 and 215. Further proof that the tetanic state which was induced was independent of the blood serum calcium is given by the behaviour of calf 220. This calf was given magnesium by injection during severe tetany; subsequently it recovered and was maintained on the deficient diet until tetany again developed. This calf was then in a tetanic state of varying severity for a period of 32 days. During this time the blood serum calcium varied from 9·0 mg./100 ml. to 12·1 mg./100 ml.,and the serum magnesium between 0·34 and 0·65 mg./100 ml. The lower values of serum calcium were associated with a particular batch of diet used for a period of 12 days during which time some alimentary disturbance occurred. There was no exacerbation of clinical signs of hyper-irritability during the

period when the serum calcium was depressed. Signs were shown most severely in fact when the blood serum concentrations of calcium were above 10 mg./100 ml.

No effect was noted on the inorganic phosphorus content of the whole blood. It appears therefore that the tetany observed was quite distinct from low calcium tetany or hypoparathyroid tetany.

Post-mortem Appearance

Nothing abnormal was observed at slaughter of calves 214, 219 and 216, none of which showed clinical signs. With calves 208, 213 and 215, the gross changes observed post mortem differed only in degree. Calf 215 was killed during the convulsive stages to demonstrate the agonal nature of the lesions observed. Calf 220 was killed in the stage of locomotor involvement 32 days after the appearance of tetany for the second time. All examinations were commenced within 30 minutes of death after exsanguination of the carcasses, with the exception of calf 213, examined several hours after death. The outstanding observation in calves 208, 213 and 215 was the presence of haemorrhagic areas coupled with acute congestion of the internal organs. These were evidently of recent origin and of a terminal (agonal) nature since in calf 215 shot during the final tonic-clonic convulsions the lesions were less severe. The heart and great vessels were particularly affected. Large haemorrhages were present in the pericardial fat and in the epicardium of the left and right ventricles. Peri-adventitial haemorrhages were present the whole length of the dorsal aorta. These were spaced regularly at intervals coinciding with the rib junctions, suggesting a terminal trauma. In one calf coagulated blood was found in the thoracic cavity and was traced to a rupture of the inferior vena cava. No internal lesions or calcified plaques were observed in the vascular system. The respiratory system was not involved, save that some recent haemorrhage between the cartilaginous rings of the trachea was observed.

In the abdominal cavity a constant finding was haemorrhage into the wall of the gall bladder and acute congestion of the liver. The kidneys were also congested. The intestinal tract likewise showed haemorrhage into the serosal coat. This consisted either of circular lines of haemorrhage embracing the circumference, or streaks, suggesting haemorrhage in the longitudinal muscle coat. Haemorrhage was also present in the perinephric fat and in the mesentery of the intestine. Gross constrictive contraction of the gut was present, particularly of the abomasum, which in one animal was divided into three lobes. Similar changes were found in the caecum and in the urinary bladder.

Examination of the muscular system revealed no signs of degenerative change, but some small areas of haemorrhage were present, and, quite understandably, extensive surface bruising. The

central nervous system of these three calves showed no obvious changes other than a fullness of the vessels.

Lesions of the vascular system clearly predominated in the calves dying in the acute convulsive stage, and as the above observations show, were clearly terminal and were most severe in unsupported organs, such as fatty tissues and the walls of the gall bladder. A photograph of the haemorrhagic heart and aorta is given in Plate V. In contradiction of the observations of Moore, *et al.* (1936, 1938), no calcification was observed.

The post mortem examination of calf 220 did not reveal any signs of recent haemorrhage save some at the base of the aorta and minute petechiae in the heart. Evidence of old haemorrhage was present in the muscular coat of the colon and scar tissue was present at the apex of the heart. There was also a bruising of the knees and metacarpus, a distinct wastage of the infraspinatus muscle, and flabbiness of the extensors of the digits. No articular abnormalities were present. The meninges showed signs of old and recent vascular accidents. The vessels of the brain were engorged, notably anterior to the corpus callosum, and at the level of mammaliary bodies. Again, no visible calcification was observed.

Histological Findings

The histological examination of the deficient calves, which was particularly thorough, revealed no lesions of significance in the muscle and in only two calves, 208 and 220, were any muscular abnormalities found. In calf 208 the cross striations were occasionally irregular or absent, and there appeared to be a rucking of the sarcolemma. This may have been due to terminal tetanic contraction affecting a small group of muscle fibres within a single muscle and may be an artefact. In calf 220 a very occasional hyaline change in the muscle fibres was found, but these were clearly not to be compared with those found in the "milk syndrome" as described by Moore, *et al.* (1936, 1938). The most outstanding lesion histologically was found in the heart and vascular tissue of the animals. Thus, in calf 208 there was thrombus formation in the small venules of the pericardial fat, associated with a necrosis of the wall, perivascular oedema and intense polymorphonuclear cell infiltration localised to the necrosed vessel. A photograph is given in Plate VI. The same lesion was found in the connective tissue of the auricle and in the wall of the ventricle and in the heart septum at the base of the inferior vena cava. Venous necrosis with polymorph reaction and interstitial haemorrhage was well marked in the myocardium of the left ventricle. Similar terminal haemorrhages were found in the coronary fat of calf 215, and in the pericardial fat and the right auricle of calf 213. It is highly probable that this lesion was terminal, as indeed was inferred from post mortem examination of calf 215. During convulsions there may be some degree of asphyxia and clearly, as

the clinical observations show, there was gross muscle incoordination and auricular fibrillation. In calf 220, which was killed following a long history of tetany, marked congestion leading to haemorrhage was also found in the ventricular septum, but there was no gross haemorrhagic change as was seen in animals which died in the terminal phase.

Sections of the kidneys showed intense congestion of the venae rectae, but no degenerative change. The costo-chondral junction was found to be quite normal. A very careful examination was made of brain and nervous tissue, involving section of the frontal, parietal and occipital parts of the cerebral cortex, the medulla, thalamus, cerebellum and different levels of the spinal cord. No unequivocal lesions were found, yet in calf 215 sections of the cortex and pons showed small haemorrhages probably of terminal origin into the brain substance. Similar minute haemorrhages were observed in calf 208. No lesions could be found in the brain of calf 213, nor in that of calf 220 though in the latter animal the meninges over the cerebellum showed pericapillary haemorrhage. No gliosis or cerebral oedema was observed, and the Purkinje cells were normal. It appears fairly certain that the small lesions that were found were terminal in origin.

Chemical Composition of Tissues

The absence of muscular lesions comparable to those observed in muscular dystrophy induced by cod-liver oil feeding was confirmed by the presence of normal amounts of creatine in samples of 5 muscles from each of the calves. Mean analytical data for the magnesium anb calcium content of the soft tissues are given in Table 3. Clearly there was no depletion of soft tissue magnesium or any pathological calcification. If the depletion was limited to the magnesium of the extracellular fluid, it would not readily be detected by analysis of the whole tissues, since the magnesium content of extracellular fluids is approximately 1/20 of that of intra-cellular material (Eichelberger and McLean, 1942). There was, however, considerable depletion of the bone magnesium in calves fed magnesium-low diets. As shown in Fig. 4 its extent appeared to be governed by the level of dietary intake, and in calves that had exhibited tetany the depletion amounted to approximately 1/3 of the bone Mg of the control animals. It appears that the Mg lost from bone is part of a body store, but it cannot be differentiated chemically from Mg remaining in the bone. At the equilibrium level the ability of the animal to mobilize Mg is apparently equalled by the avidity of the bone salt for Mg (Duckworth and Godden, 1941). The increase in the Ca content of the bone of deficient calves is probably due to the replacement of mobilized Mg by Ca (Duckworth, Godden and Warnock, 1940). In calf 220, which was in a state of chronic tetany for thirty days, the bone Mg was lowered to 1/3 of the normal value and there was no concomitant increase in Ca content.

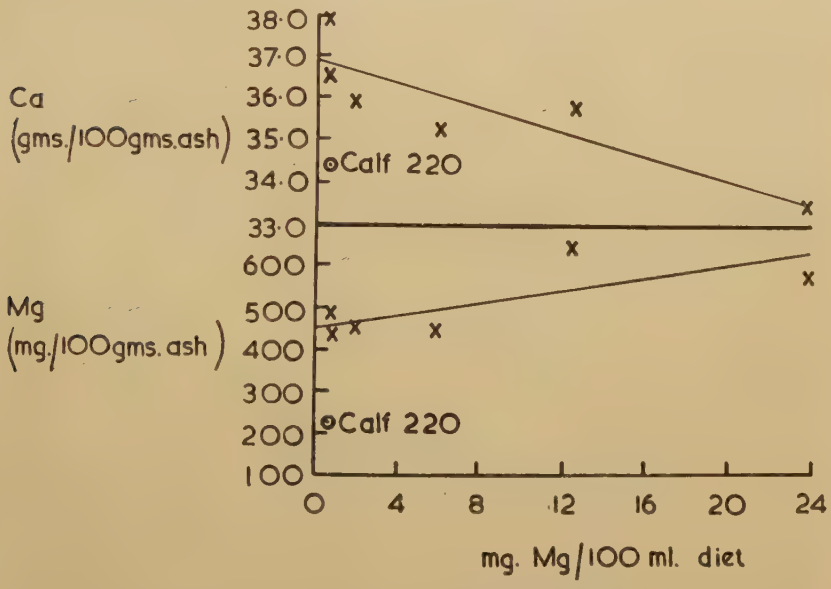
TABLE 3.
THE CALCIUM AND MAGNESIUM CONTENT OF THE SOFT TISSUES OF NORMAL AND DEFICIENT CALVES*.

Soft tissue	*Calcium (mg./100 g.)*		*Magnesium (mg./100 g.)*			
	Normal	*Deficient*	*Normal*		*Deficient*	
Supraspinatus muscle ..	7·1	6·6	18·4		19·2	
Infraspinatus muscle ..	6·5	6·4	18·0		15·9	
Rectus femoris muscle ..	5·7	8·8	20·2	18·9	18·5	18·7
Cardiac muscle	10·4	8·1	19·0		21·3	
Diaphragm muscle ..	11·5	12·7	19·1		18·8	
Kidney	14·3	10·3	13·4		13·8	
Spleen	13·7	9·1	17·2		14·3	
Lung	15·8	17·9	3·6		3·2	
Liver	8·0	6·6	10·4		15·4	
Brain	10·5	9·1	9·7		11·2	
Dorsal aorta wall	25·0	18·5	5·3		5·1	

*Normal calves, are nos. 214 and 219 and one normal week-old calf given an adequate diet.

Deficient calves, are nos. 208, 213, 215 and 220 which showed severe clinical signs, including gross tetany.

Fig. 4.



The calcium and magnesium content of the bones of calves at death in relation to the dietary concentration of magnesium. Each point represents a mean value—derived from analyses of rib, fore cannon and hind cannon of an individual animal. Calf 220 was maintained on a magnesium deficient diet for 30 days after it first exhibited tetany.

DISCUSSION

The pathology of experimental Mg deficiency in the calf as observed in the present studies differs in many respects from that of the so-called "milk syndrome". In particular, it has not been possible to demonstrate any pathological calcification of the tissues or formation of calcified plaques in the aorta and endocardium even in animals which have exhibited tetany and have been maintained on deficient diets for long periods. It appears also that the hyaline degeneration of the muscles observed by Moore, *et al.* (1936, 1938) was not due to a deficiency of Mg or interference with Mg metabolism but to some other nutritional failures. Possibly vitamin E deficiency was involved.

The massive haemorrhage observed in the deficient calves has been reported to occur in cows dying as a result of lactation tetany, a disease in which the blood serum Mg is lowered (Blakemore and Stewart, 1932-33). However, the haemosiderosis of the spleen reported by these workers was not observed in the present study. Renal damage, which is an early lesion in the Mg-deficient rat (Cramer, 1932; Greenberg, Lucia and Tufts, 1938) was not noted in any of the deficient calves, but the terminal venous necrosis in the heart and vascular system agree well with observations made with rats by Greenberg, Anderson and Tufts (1936) and by Lowenhaupt, Schulman and Greenberg (1950). It has been claimed by several workers, that, in other species, notably in chicks (Bird, 1949) and rats (Barron, Brown and Pearson, 1949) lesions characterised by tigrolysis, nuclear alterations and failure of the dendrites to stain occur in the cerebellar Purkinje cells, but no such changes occur in the calf.

The aetiology of the tetany of Mg deficiency is obscure. The fact that the Mg content of the soft tissues remains within the normal range suggests that in the tissue cells there is probably ample Mg for the activation of those enzyme systems concerned with carbohydrate metabolism which need Mg or Mn for activation. It has been suggested by Greville and Lehmann (1943) that the hyper-irritability of muscle in tetany arises directly in the muscle cell due to Mg^{++}— Ca^{++} antagonism in the activation of adenosine triphosphatase. A greater breakdown of adenosine triphosphate, with exaggerated response to motor stimuli might thus occur as the result of Mg deprivation. The fact that the total Mg and the total Ca content of the muscle is unchanged in the deficiency does not make this supposition likely, and it would appear that the tetany is not primarily of muscle cell origin. The absence of depletion of Mg in nervous tissue and the absence of neurological lesions suggest the tetany is not due to a primary neurological change. It must be pointed out, however, that some of the clinical signs observed are closely comparable to those seen in vitamin B_1 deficiency. Since Mg

is concerned in the activation of those enzymes which have thiamine as the prosthetic group, this aspect needs further study.

The greatest depletion of Mg in the deficiency takes place in the blood serum, and it is legitimate to suppose that a similar depletion occurs in other extracellular fluids. Recent work by del Castello and Engbaek (1953) has shown that Mg^{++} plays an important part in the transmission of impulses at the neuromuscular junction. A low concentration of Mg or more particularly a low Mg^{++}/Ca^{++} ratio potentiates the release of acetyl choline and the transmission. It is possible that low concentrations of Mg^{++} in the extracellular fluid surrounding the muscle end-plates may produce tetany through this mechanism.

The depletion of the bones of the calves is comparable to the depletion observed in Mg-deficient rats (Duckworth and Godden, 1941; Duckworth, Godden and Warnock, 1940) and represents a loss of rather labile body stores. As far as tetany is concerned this loss is of no immediate biochemical significance.

SUMMARY

Magnesium deficiency has been produced in calves by feeding them an artificial diet containing only 0.5 mg./100 ml. but adequate in all other respects.

The clinical signs and the gross and histological pathology of the disease are described in detail.

The disease was associated with a fall in the blood serum Mg to values below 0·5 mg./100 ml., and it was shown that to maintain a normal serum value of 2·1 mg./100 ml. a dietary concentration of Mg of 14 mg./100 ml. was necessary.

There was no detectable loss of Mg from the soft tissues of the body, but bone Mg was depleted to the extent of 30 per cent.

The results are discussed in relation to the so-called "milk syndrome". It appears that uncomplicated Mg deficiency in calves aged 50 to 120 days is not associated with the pathological calcification of the endocardium and vascular system observed in the former disease.

ACKNOWLEDGMENTS

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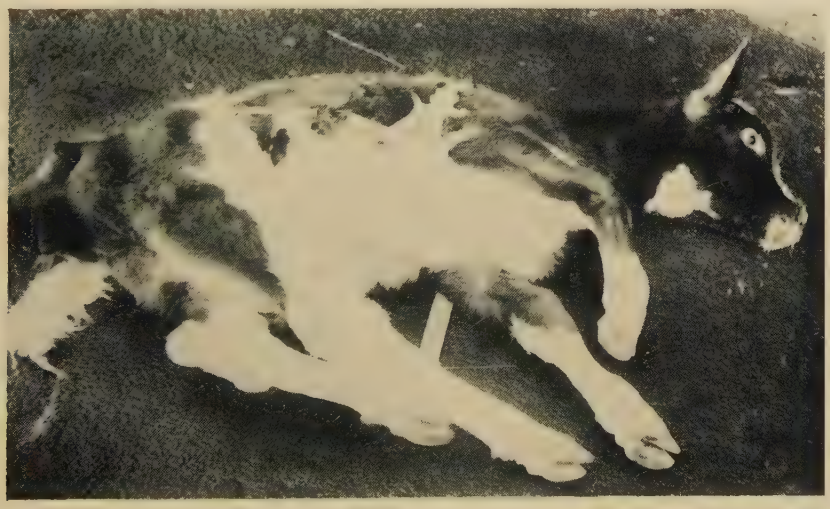
Plate I.

Opisthotonus exhibited by calf no. 208 given a diet containing 0·5 mg. magnesium/100 ml. The photograph was taken 3 days prior to death in convulsions.

Plate II.

The change in the carriage of the head and ears of calf no. 215, given a diet containing 0·5 mg. magnesium/100 ml. The photograph was taken 14 days prior to death in convulsions.

Plate III.



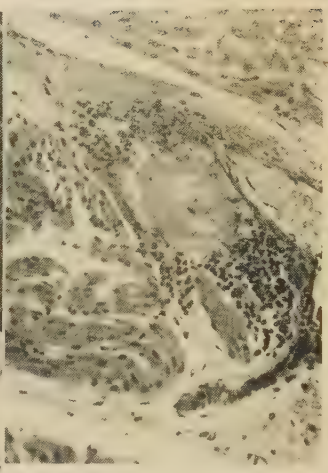
Terminal convulsions of calf no. 215, given a diet containing 0·5 magnesium/100 ml.

To face page 175

Plate IV.

The abnormality of gait and relaxation of the metacarpal joint seen in calf 220 following recovery from tetany.

Plate VI.



Histological Section of the site of a haemorrhage in the wall of the right auricle of the heart of Calf no. 208. The ruptured vessel may be seen together with a large extra-vascular fibrin clot around which are massed polymorphonuclear leucocytes. Extra capillary erythrocytes may be seen in profusion between the muscle bundles. Haemotoxylin eosin preparation.

Plate V.

The heart and part of the dorsal aorta of calf no. 213 given a diet containing 1·6 mg. magnesium/100 ml. Note the massive terminal haemorrhage.

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EXPERIMENTAL MAGNESIUM DEFICIENCY IN CALVES

2. The Metabolism of Calcium, Magnesium and Nitrogen and Magnesium Requirements

By

K. L. Blaxter and J. A. F. Rook

Hannah Dairy Research Institute, Kirkhill, Ayr

INTRODUCTION

Few studies of the excretion and body storage of magnesium have been made with cattle in relation to the development of tetany. Ray (1942) has shown that when tetany occurs in cattle, negative balances of magnesium are found, and that magnesium supplements give rise to positive balances and abolish the tetany.

No estimates of the magnesium requirements of cattle are available apart from a single value of 400 mg./day inferred from studies of blood analysis (Huffman, Conley, Lightfoot and Duncan, 1941). As previously pointed out, this value is very considerably greater than estimates of the minimal requirements of other species (Blaxter, Rook and MacDonald, 1953).

The present experiments were undertaken to study the factors which affect body storage of magnesium in calves and to provide estimates of their minimal requirements.

EXPERIMENTAL PROCEDURE

Animals and diets. The experimental diets and the clinical history of the seven animals used as experimental subjects have been described in the previous paper (Blaxter, *et al.*, 1953).

Metabolic methods. The experimental diets were weighed or measured by volume and the faeces and urine from each calf were collected quantitatively each day. Samples of the excreta and the food of each calf were pooled over four day periods. Contamination of urine with faeces occurred on three occasions only, when the calves concerned suffered from a transitory diarrhoea. The balance of intake and excretion was, however, unaffected since collection of excreta was complete.

Analytical methods. The samples of food and excreta were analysed for nitrogen by the macro-Kjeldahl procedure. Phosphorus was determined by the method of Fiske and Subbarow (1925), following wet ashing, and calcium and magnesium were determined on ashed samples as previously described (Blaxter, *et al.*, 1953). Very large samples of urine were required for the accurate estimation of the minute amounts of magnesium excreted by this route. Faecal dry matter was determined by direct drying at 100°C.

RESULTS

The Interpretation of Data on Magnesium Retention

Growth, measured simply as the gain in body weight per unit time, is the sum of the rates of retention of many dietary ingredients. These individual rates differ very markedly one from another, since they reflect developmental changes involving different demands for structural components. The correct interpretation of balances of a particular element can only be made when they are related to the simultaneous rates of accumulation of other body constituents.

A study was therefore made of the relationship between the retention of magnesium and the simultaneous retentions of N, Ca and P to provide a basis for the interpretation of magnesium retention in the individual. During growth, magnesium is retained in the soft tissues and in the bones associated with the apatite of the bone salt (Shohl, 1939). From a consideration of our own analyses and those of Watchorn and McCance (1937) and of Cunningham (1936), it was found that the ratio of N : Mg in the soft tissues and of Ca : Mg in bone are approximately constant giving mean values of 140 : 1 and 45 : 1 respectively.

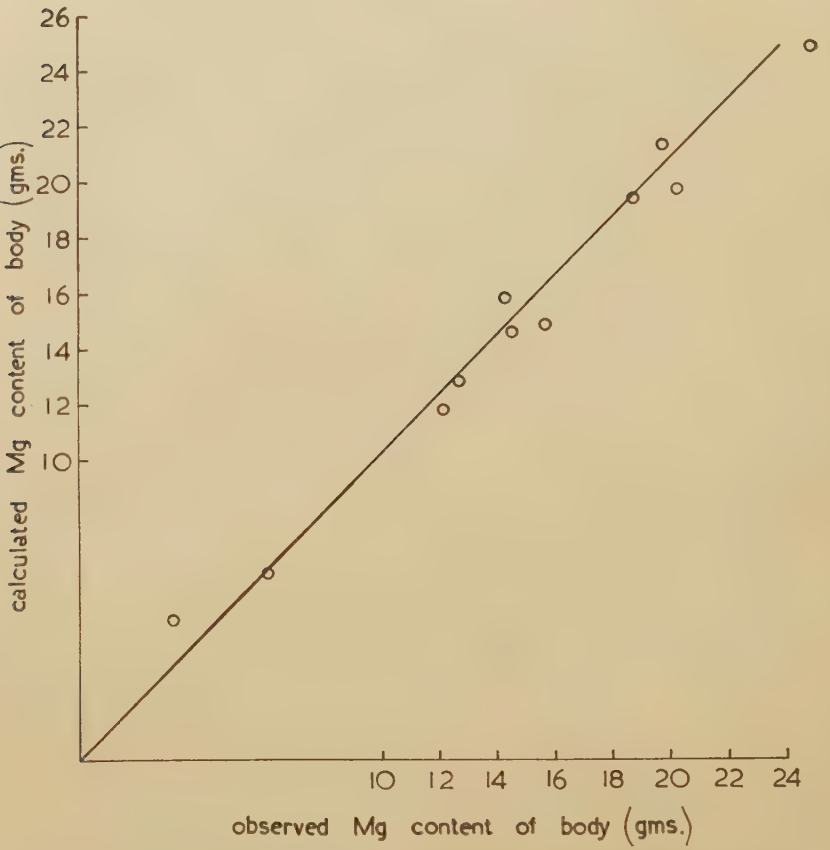
These two ratios permit the estimation of "normal" magnesium retention from concomitant data relating to the retention of N and Ca. The accuracy of prediction was checked by applying them to analytical data obtained on the complete bodies of calves (Hogan and Nierman, 1927; Haigh, Moulton and Trowbridge, 1920). As shown in Fig. 1 the agreement between the observed magnesium contents of the bodies of these calves and those computed was good. A further demonstration of the validity of the two ratios is given in Fig. 2. This shows for a normal calf, 214, the cumulative retention of Mg obtained from the balance experiments and the cumulative retention calculated from the balances of Ca and N. Over a period of 54 days the discrepancy was very slight.

The Effect of Magnesium Deficiency on N and Ca Retention

Comparisons of the rates of retention of Ca and N by animals given rations adequate and inadequate in magnesium may be made at two stages in the development of the deficiency syndrome, firstly in the period when no clinical signs are apparent though the animal is consuming the deficient diet, and secondly when clinical signs have developed. Table 1 shows the mean N and Ca balances of the calves when given 4 l. diet daily at the "pre-clinical" stage. There were no differences of any significance.

In Table 2 a comparison is made of the N and Ca retention of affected and normal calves. Calf 213 was not included since diarrhoea coincided with the terminal phase. The Ca and N retention were not affected by the deficiency state.

Fig. 1.



The accuracy of prediction of total body magnesium from simultaneous estimates of total body calcium and nitrogen, using the ratios Ca: Mg, 45: 1 and N: Mg, 140:1. The data are those of Hogan & Nierman (1927) and Haigh, Moulton & Trowbridge (1920).

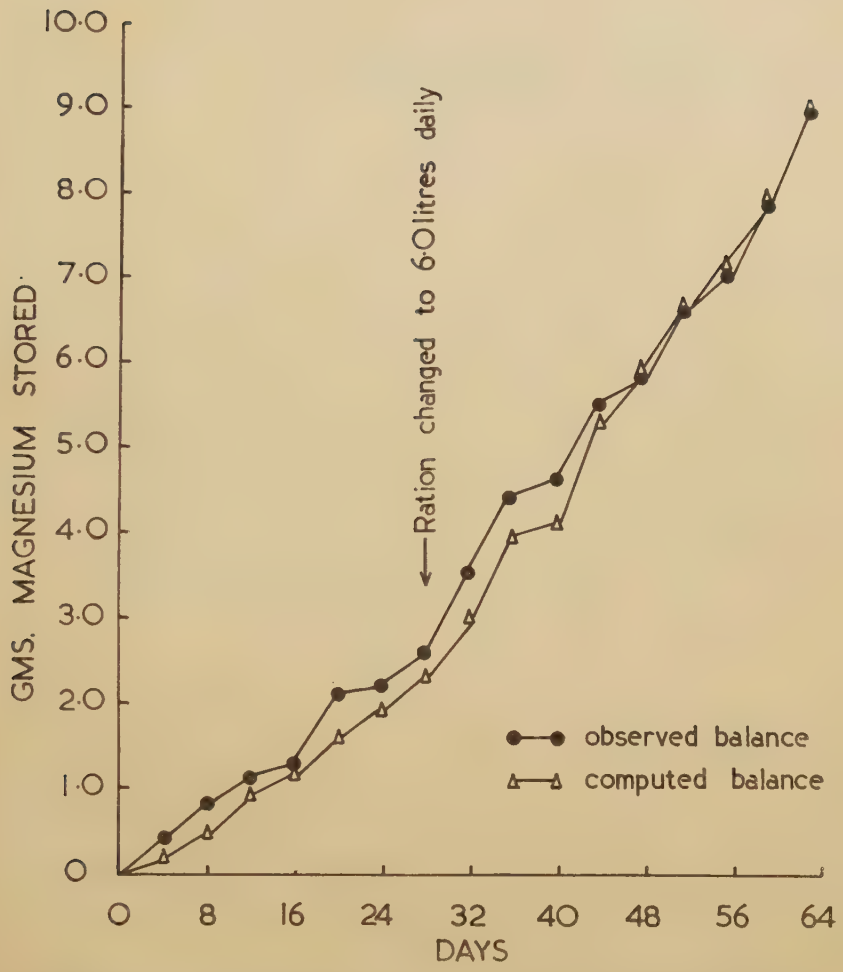
TABLE 1

MEAN DAILY RETENTION BY THE CALVES OF CA AND N

Prior to development of clinical signs

	Calves which subsequently showed no signs of tetany			*Calves which subsequently developed severe tetany*			
Calf No.	219	214	216	213	215	208	220
Daily intake of Mg (mg.)	776	549	239	69	19	21	20
Daily retention of N (g.)	+3·56	+4·78	+4·99	+5·18	+5·23	+7·94	+5·30
Daily retention of Ca (g.)	+3·36	+2·37	+1·80	+1·68	+2·64	+2·28	+3·66

Fig. 2.



The actual retention of magnesium in the body of calf 214 given the normal diet compared with the retention computed from data on its Ca and N metabolism.

TABLE 2

THE N AND CA RETENTION

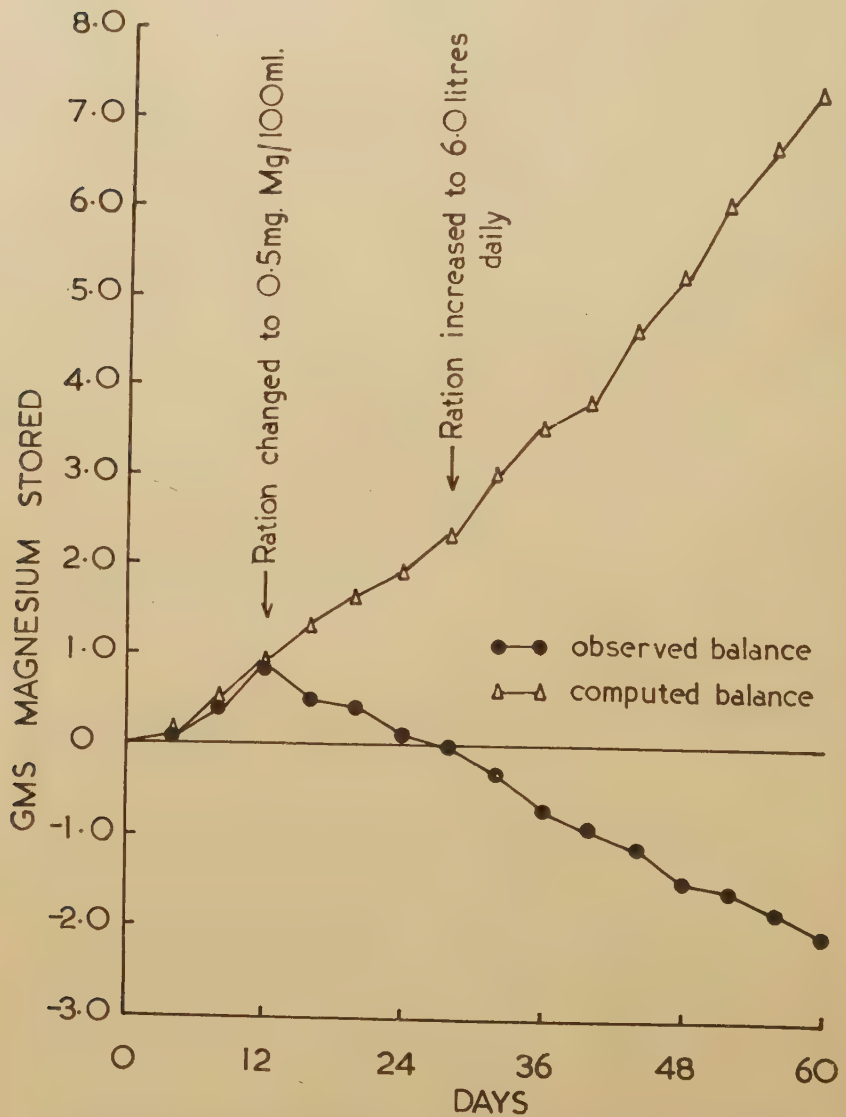
Calves showing clinical signs of deficiency compared with that of control calves under comparable conditions

Affected calves	*N retention (g./day)*	*Ca retention (g./day)*	*Normal calves*	*N retention (g./day)*	*Ca retention (g./day)*
215	8·75	3·44	214	9·46	3·47
208	11·20	3·28	214	11·98	3·40
220	24·18	—	219	22·11	—

The Extent of the Magnesium Depletion Necessary to Cause Fatal Signs

Fig. 3 gives data for the deficient calf 215. This shows that transference to the magnesium-low diet resulted in a reduction of magnesium retention, and a progressive depletion of the animal's

Fig. 3.



The actual retention of magnesium in the body of calf 215 given the diet containing 0·5 mg. Mg/100 ml. compared with the retention computed from data on its Ca and N metabolism.

body magnesium. The results for all the calves given rations low in magnesium are exactly comparable and show that the cumulative deficiency of Mg in the body was of the order of 9 g. when death occurred. From the regression of total body content of magnesium on body weight, it may be calculated that the body of calf 215 should have contained 19 g. at the commencement of the experiment. At death the loss from its body, calculated from balances of intake and of excretion, had been 2 g., giving an estimate of 17 g. total body magnesium at death. From the calcium and nitrogen retentions, it was calculated that the calf should have retained 7 g., giving, at death, an expected total body magnesium of 26 g. The percentage depletion of the magnesium content of the animal was thus 35 per cent.

Carcase analyses indicated a depletion of the magnesium content of bone by 45 to 50 per cent and no loss of soft tissue magnesium (Blaxter, *et al.* 1953). Since approximately 60 per cent of the body magnesium is present in the bones of the calf, these results indicate that the depletion of the total body magnesium was approximately 30 per cent. This estimate is in reasonable agreement with the value of 35 per cent arrived at by balance methods as detailed above.

Comparable calculations for other calves gave similar results, all suggesting that a depletion of body magnesium of 30 per cent or more was necessary to cause fatal convulsions.

The Effect of Diarrhoea on Magnesium Retention

From Fig. 4, which shows cumulative magnesium balance data for calf 216, it is evident that mild diarrhoea resulted in a depression of the magnesium retention. Comparable results were obtained with calf 213. For three calves suffering from diarrhoea and given deficient diets, the mean daily loss of magnesium from the body was 117 mg. In the same calves when fully recovered the loss was only 42 mg., the difference being highly significant statistically. This increased loss of magnesium during diarrhoea does not, however, specifically accelerate the depletion of the calf, since the retention of nitrogen and calcium is simultaneously depressed.

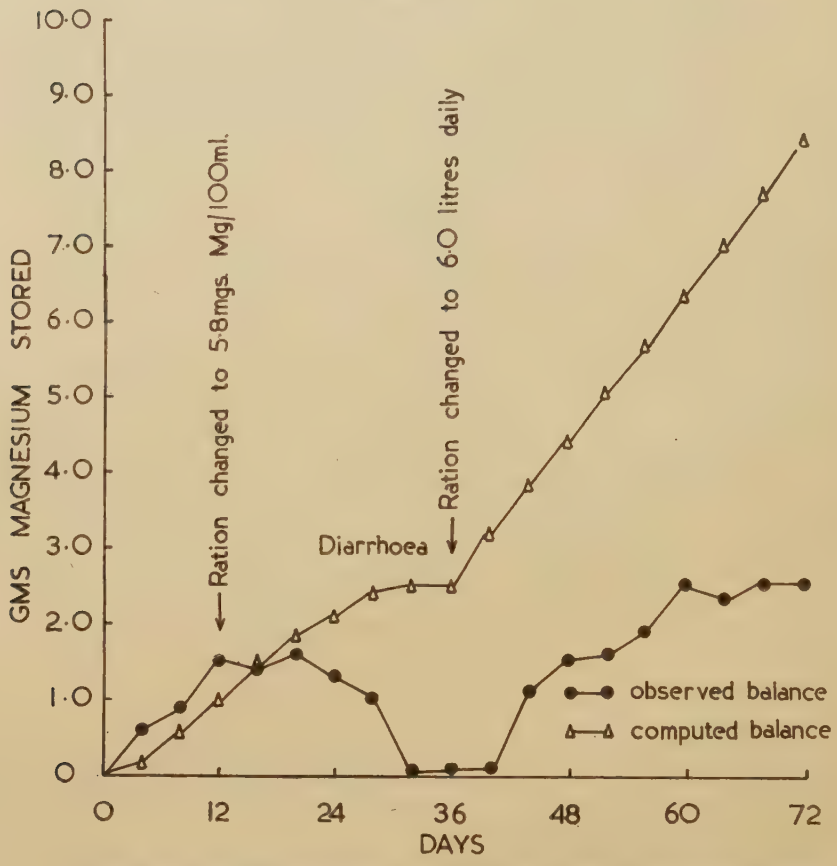
The Relation between Magnesium Retention and Magnesium Intake

In Fig. 5 data are shown for the magnesium balances obtained when 6·0 l. of diet were given. It will be noted that as the magnesium content of the ration was increased, so the magnesium retention increased at an ever decreasing rate. The exponential curve

$$R = 217 - 326e^{-0{\cdot}00269\,I}$$

where R = Mg retention in mg./day, and I = intake in mg./day was

Fig. 4.



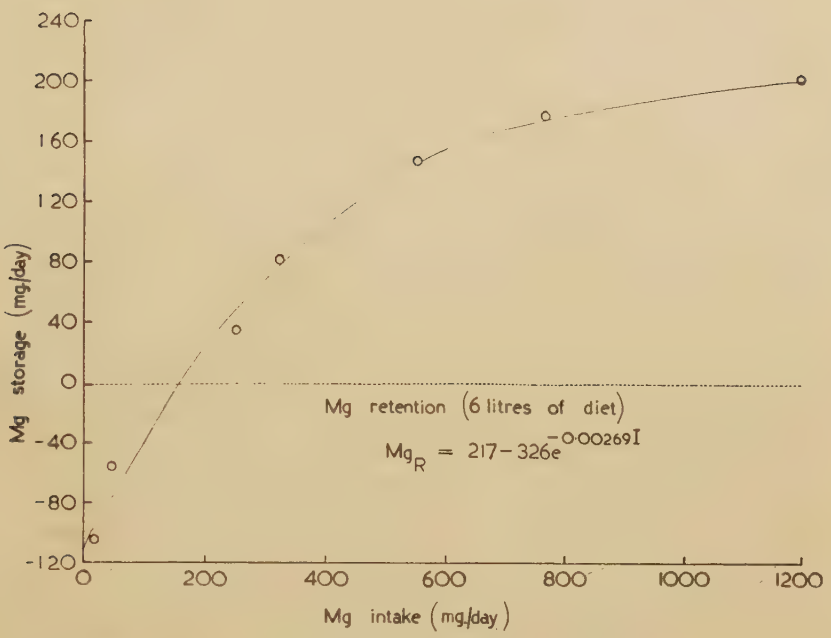
The actual retention of magnesium by calf 216 given the diet containing 5·8 mg. Mg/100 ml., compared with the retention computed from data on its Ca and N metabolism. Note that diarrhoea depresses both the actual retention and that computed.

fitted to these data and is the curve shown. This curve indicates that when no magnesium was included in the ration, then the loss of magnesium from the body was 109 mg./day and that the maximal daily retention of magnesium obtainable by increasing the concentration of magnesium would be 217 mg./day. Similar statistical calculations with calves given 4·0 l., indicated the maximal retention to be 110 mg./day. The equation was

$$R = 110 - 219e^{-0.00376\,I}$$

The amount of dietary magnesium necessary to give a retention 90 per cent of the maximum was calculated to be 1007 mg. when 6·0 l. diet were supplied and 755 mg. when 4·0 l. were given. These

Fig. 5.



The daily retention of magnesium by calves in relation to the daily intake in the diet. These calves all received 6 litres of diet daily.

figures correspond to dietary concentrations of magnesium of 16·8 mg. Mg/100 ml. and 18.8 mg. Mg/100 ml. respectively, the former being the more precise estimate. This value is an approximation to the dietary needs of the calf in terms of magnesium, and is considerably above the content of magnesium present in cow's milk (7 to 10 mg./100 ml.). From the above equation it may be calculated that cow's milk, containing a mean of 9 mg./100 ml. would result in the retention of only 141 mg., that is 65 per cent of the maximal value. These results suggest therefore that when whole milk is the sole diet, its magnesium content is a factor limiting magnesium retention.

Maintenance Requirements and Growth Requirements

The loss of magnesium from the body when there is no magnesium in the diet is a valid estimate of the net requirement of magnesium for maintenance; it represents the inevitable loss of magnesium in the urine and faeces. From the analyses of the data given above, this net loss has been shown to be 109 mg. for calves given 6·0 l. of diet and 109 mg. also for those given 4·0 l. These values do not take into account the losses associated with the utilization of dietary magnesium which may be obtained from the

exponential equations, by computing the requirement of magnesium to give magnesium equilibrium. These requirements are 152 mg. and 183 mg. magnesium for 6 l. and 4 l. of diet respectively, suggesting that the percentage utilization of dietary magnesium at magnesium equilibrium is low—somewhere between 59 and 71 per cent. Such a percentage retention is considerably lower than the percentage retention of calcium by the growing calf (Blaxter and Wood, 1952).

Requirements for growth clearly depend on the relative proportions of bone, soft tissues and fat deposited. These will presumably vary with rate of growth. A tentative estimate of the net requirement is 700 mg. magnesium/kg. of weight gained. The amount needed in the diet to meet this requirement would be considerably greater.

It may be pointed out that since the calf requires only 2·3 to 2·5 l. of milk for body weight maintenance, the dietary concentration needed is at the maximum 8·0 mg. magnesium/100 ml., whereas when the intake of food is 6·0 l. a day, permitting a gain of over 400 g. daily, the requirement is as high as 16 mg./100 ml. It follows that very rapid growth on whole milk would probably result in a greater depletion of the total body magnesium than slow growth, where the dietary intake is but little above maintenance levels. Rapid growth would intensify magnesium requirements when these are expressed in terms of a dietary concentration.

Routes of Excretion of Magnesium

Table 3 gives typical results which indicate that the amount of

TABLE 3

THE EXCRETION OF MAGNESIUM IN THE URINE AND FAECES

Calves given various amounts of magnesium

Calf no.	*Daily amount of diet* (l.)	*Magnesium metabolism, mg./day**			
		Intake	*Excretion in*		*Balance*
			Urine	*Faeces*	
214	6	606	17	431	+158
216	6	307	9	236	+ 62
213	6	122	2	153	— 32
215	6	24	4	76	— 56
214	4	553	14	463	+ 76
208	4	19	3	60	— 44

*The figures refer to mean balances over periods of 8 days only, not the whole experimental periods.

magnesium excreted in the urine is always small relative to that excreted in the faeces. When the dietary concentration is high, as much as 17 mg. out of 448 are excreted in the urine, but low intakes are invariably associated with the excretion of only a few mg. daily. The urinary excretion of magnesium accounts in these instances for about 5 per cent of the total excretion. Even at low levels of intake when the total excretion exceeds the total intake, the major channel of loss is the faeces. The concentration of magnesium in the faecal dry matter of the calves given the diet containing 0.5 mg. magnesium/100 ml. was 170 to 200 mg./100 g., the calcium content being 4 to 6 per cent. There was no apparent relationship between the amount of dry faeces excreted and the magnesium concentration in the range of faecal weight studied. Undoubtedly the presence of these large quantities of magnesium in the faeces means that magnesium secreted into the intestine in intestinal juices is not reabsorbed with ease. That difficulty of reabsorption may be an important factor receives support from the fact that the absorption of dietary magnesium is also not particularly efficient as may be inferred from the previous discussion of maintenance requirements.

DISCUSSION

The above results show that the requirement of magnesium by the growing calf is approximately 16 to 18 mg./100 ml. diet, when that diet simulates whole milk in almost all respects. This estimate applies to calves making limited growth, and it is suggested that since requirements at body weight maintenance are met by milk containing 8 mg./100 ml., that very rapid growth would call for even higher dietary concentrations of magnesium. These estimates are higher than those previously given from a consideration of the data relating to the concentration of magnesium in the blood serum of these calves, but not grossly so. There the assumption was made that the maintenance of a serum concentration of 2·15 mg. could be regarded as a criterion of normality, and the dietary concentration of magnesium necessary for this was found to be 13 to 14 mg. The difference between these estimates reflects the many difficulties which accrue when attempts are made to assess normality. If absence of clinical signs in 50 days were taken as the sole criterion, then a dietary concentration of only 3 to 5 mg. would be sufficient. Maintenance of a particular blood concentration of magnesium or of a maximal retention rate of magnesium are, however, clearly superior measures of dietary adequacy, and the present results suggest that calves given rations of large amounts of whole cow's milk do not receive sufficient magnesium to ensure saturation of their bones and soft tissues with magnesium. This conclusion agrees with those obtained by Huffman and his collaborators (1941); the present results, however, place dietary requirements much lower than they have suggested.

SUMMARY

Continuous metabolic studies were made with calves given rations containing different amounts of magnesium.

Studies of the cumulative retention of magnesium showed that in calves given normal amounts of magnesium, retention could be predicted from simultaneous data on calcium and nitrogen retention.

Magnesium deficiency had no effect on calcium metabolism or nitrogen metabolism.

It was shown that it was necessary to deplete the calf of 30 per cent of its total body magnesium to cause tetanic convulsions.

Diarrhoea accelerated the magnesium loss, but calcium and nitrogen retention were similarly depressed.

It was calculated that at rates of gain of 1 lb./day the calf requires a dietary concentration of 16 to 18 mg. magnesium 100 ml. to ensure 90 per cent saturation of its capabilities to retain magnesium from a standard amount of diet.

The maintenance requirement for magnesium was met by an intake of 150 to 180 mg. magnesium/day, equivalent to a dietary concentration of approximately 8 mg./100 ml. or a requirement per kg. of body weight of approximately 40 mg.

The percentage retention of dietary magnesium was low, large quantities being lost in the faeces. The loss in the urine was always very small.

The results are discussed in relation to magnesium requirements.

ACKNOWLEDGMENTS

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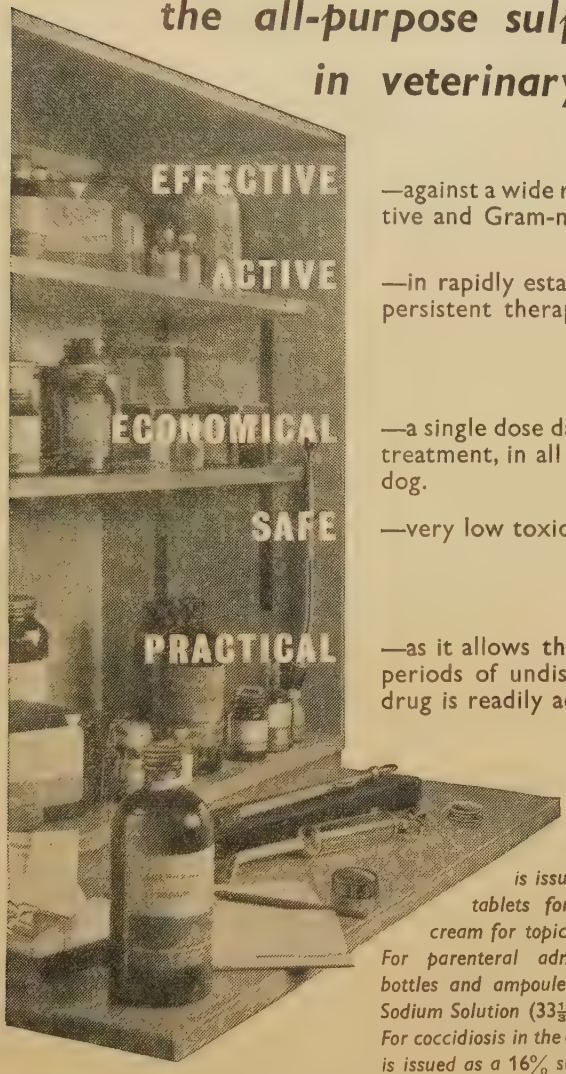

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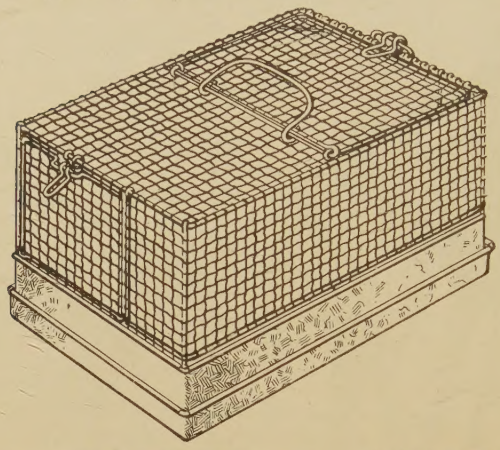

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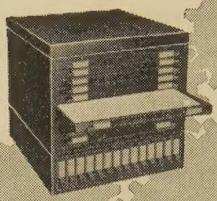

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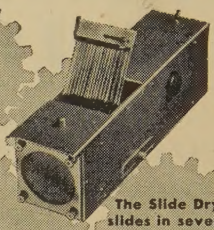

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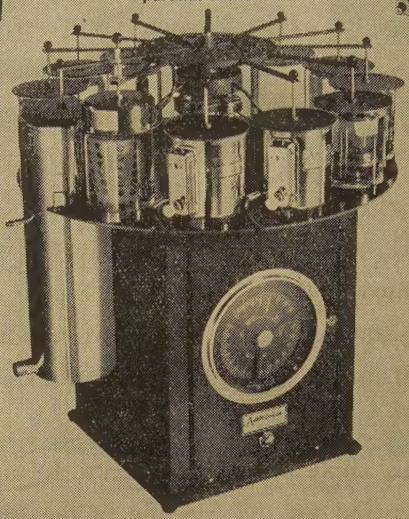

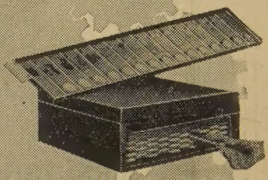

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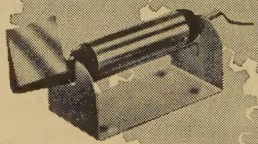

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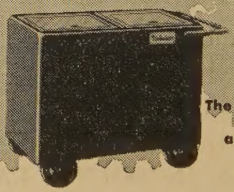

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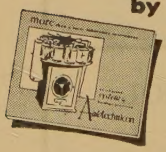

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